PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/00

A2

(11) International Publication Number: **WO 99/06068**

(43) International Publication Date: 11 February 1999 (11.02.99)

(21) International Application Number: PCT/US98/15447

(22) International Filing Date: 27 July 1998 (27.07.98)

(30) Priority Data:
08/904,227 31 July 1997 (31.07.97) US

(71) Applicant: HAWAII BIOTECHNOLOGY GROUP, INC. [US/US]; Suite 236, 99–193 Aiea Heights Drive, Aiea, HI 96701 (US).

(72) Inventors: IVY, John, M.; 1104 Rio Bravo Court, College Station, TX 77845 (US). PETERS, Iain, D.; 2680 East Manoa Road #A3, Honolulu, HI 96822 (US). COLLER, Beth–Ann, G.; 98–1781C Kaahumanu Street, Aiea, HI 96701 (US). MCDONNELL, Michael; 204 Paiko Drive, Honolulu, HI 96821 (US). HARADA, Kent, E.; 1084 Maluna Street, Honolulu, HI 96818 (US).

(74) Agents: MURASHIGE, Kate, H. et al.; Morrison & Foerster LLP, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006–1888 (US).

(81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CU, CZ, EE, FI, GE, GH, GM, HR, HU, ID, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT DIMERIC ENVELOPE VACCINE AGAINST FLAVIVIRAL INFECTION

(57) Abstract

The present invention discloses and claims vaccines containing, as an active ingredient, a secreted recombinantly produced dimeric form of truncated flaviviral envelope protein. The vaccines are capable of eliciting the production of neutralizing antibodies against flaviviruses. The dimeric forms of truncated flaviviral envelope protein are formed 1) by directly linking two tandem copies of 80 % E in a head to tail fashion via a flexible tether; 2) via the formation of a leucine zipper domain through the homodimeric association of two leucine zipper helices each fused to the carboxy terminus of an 80 % E molecule; or 3) via the formation of a non–covalently associated four–helix bundle domain formed upon association of two helix–turn–helix moieties each attached to the carboxy terminus of an 80 % E molecule. All products are expressed as a polyprotein including prM and the modified 80 % E products are secreted from *Drosophila melanogaster* Schneider 2 cells using the human tissue plasminogen activator secretion signal sequence (tPA_L). Secreted products are generally more easily purified than those expressed intracellularly, facilitating vaccine production. One embodiment of the present invention is directed to a vaccine for protection of a subject against infection by dengue virus. The vaccine contains, as active ingredient, the dimeric form of truncated envelope protein of a dengue virus serotype. The dimeric truncated E is secreted as a recombinantly produced protein from eucaryotic cells. The vaccine may further contain portions of additional dengue virus serotype dimeric E proteins similarly produced. Another embodiment of the present invention is directed to methods to utilize the dimeric form of truncated dengue envelope protein for diagnosis of infection in individuals at risk for the disease. The diagnostic contains, as active ingredient, the dimeric form of truncated envelope protein of a dengue virus serotype. The dimeric truncated E is secreted as a recombinantly produced protein from eucaryotic cells. The diagnostic may further contain portions of additional dengue virus serotype dimeric E proteins similarly produced.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

RECOMBINANT DIMERIC ENVELOPE VACCINE AGAINST FLAVIVIRAL INFECTION

Technical Field

This invention relates to protection against and diagnosis of flaviviral infection. More specifically, the invention concerns recombinantly produced dimers of truncated flaviviral envelope protein secreted as mature proteins from eucaryotic cells and which induce high titer virus neutralizing antibodies believed to be important in protection against flaviviral infection and which are useful in diagnosis of infection by the virus.

Background Art

The four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) belong to the family *Flaviviridae* which also includes the Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF). Flaviviruses are small, enveloped viruses containing a single, positive-strand, genomic RNA. The envelope of flaviviruses is derived from the host cell membrane and is decorated with virally-encoded transmembrane proteins membrane (M) and envelope (E). While mature E protein and the precursor to M, prM, are glycosylated, the much smaller mature M protein is not. The E glycoprotein, which is the largest viral structural protein, contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. It is also a major target of the host immune system, inducing virus neutralizing antibodies, protective immunity, as well as antibodies which inhibit hemagglutination.

Dengue viruses are transmitted to man by mosquitoes of the genus *Aedes*, primarily *A. aegypti* and *A. albopictus*. The viruses cause an illness manifested by high fever, headache, aching muscles and joints, and rash. Some cases, typically in children, result in a more severe forms of infection, dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), marked by severe hemorrhage, vascular

permeability, or both, leading to shock. Without diagnosis and prompt medical intervention, the sudden onset and rapid progression of DHF/DSS can be fatal.

Flaviviruses are the most significant group of arthropod-transmitted viruses in terms of global morbidity and mortality with an estimated one hundred million cases of dengue fever occurring annually (Halstead, 1988). With the global increase in population and urbanization especially throughout the tropics, and the lack of sustained mosquito control measures, the mosquito vectors of flavivirus have distributed throughout the tropics, subtropics, and some temperate areas, bringing the risk of flaviviral infection to over half the world's population. Modern jet travel and human emigration have facilitated global distribution of dengue serotypes, such that now multiple serotypes of dengue are endemic in many regions. Accompanying this in the last 15 years has been an increase in the frequency of dengue epidemics and the incidence of DHF/DSS. For example, in Southeast Asia, DHF/DSS is a leading cause of hospitalization and death among children (Hayes and Gubler, 1992).

The flaviviral genome is a single strand, positive-sense RNA molecule, approximately 10,500 nucleotides in length containing short 5' and 3' untranslated regions, a single long open reading frame, a 5' cap, and a nonpolyadenylated 3' terminus. The complete nucleotide sequence of numerous flaviviral genomes, including all four DEN serotypes and YF virus have been reported (Fu et al., 1992; Deubel et al, 1986; Hahn et al, 1988; Osatomi et al., 1990; Zhao et al, 1986; Mackow et al., 1987; Rice et al., 1985). The ten gene products encoded by the single open reading frame are translated as a polyprotein organized in the order, capsid (C), premembrane/membrane (prM/M), envelope (E), nonstructural protein (NS) 1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (Chambers, et al. 1990). Processing of the encoded polyprotein is initiated cotranslationally, and full maturation requires both host and virally-encoded proteases. The sites of proteolytic cleavage in the YF virus have been determined by comparing the nucleotide sequence and the amino terminal sequences of the viral proteins. Subsequent to initial processing of the polyprotein, prM is converted to M during viral release (Wengler, G. et al., 1989. J Virol 63:2521-

2526) and anchored C is processed during virus maturation (Nowak et al., 1987. Virology 156:127-137).

While all dengue viruses are antigenically related, antigenic distinctions exist which define the four dengue virus serotypes. Infection of an individual with one serotype does not apparently provide long-term immunity against the other serotypes. In fact, secondary infections with heterologous serotypes are becoming increasingly prevalent as multiple serotypes co-circulate in a geographic area. In general, primary infections elicit mostly IgM antibodies directed against type-specific determinants. On the other hand, secondary infection by a heterologous serotype is characterized by IgG antibodies that are flavivirus crossreactive. Dengue virus vaccine development is complicated by the observation that immunity acquired by infection with one serotype may in fact enhance pathogenicity by dengue virus of other types. Halstead (1982) demonstrated that anti-dengue antibodies can augment virus infectivity *in vitro*, and proposes that serotype crossreactive, non-neutralizing antibodies to E enhance infection *in vivo*, resulting in DHF/DSS (Halstead, 1981). This viewpoint is not however, universally accepted (Rosen, 1989). For example, Kurane et al (1991) proposed that dengue serotype-crossreactive $CD4^+$ $CD8^-$ cytotoxic T cells (CTLs) specific for NS3 may contribute to the pathogenesis of DHF/DSS by producing IFN-γ and by lysing dengue virus-infected monocytes. Recent evidence demonstrating that CTLs specific for E are not serotype-crossreactive may suggest that use of E subunit vaccines would not induce the potentially harmful cross-reactive CTL response (Livingston et al, 1994). Regardless of the mechanism for enhanced pathogenicity of a secondary, heterologous dengue viral infection, strategies employing a tetravalent vaccine should avoid such complications. Helpful reviews of the nature of the flaviviral diseases, the history of attempts to develop suitable vaccines, and structural features of flaviviruses in general as well as the molecular structural features of the envelope protein of flaviviruses are available (Halstead 1988; Brandt 1990; Chambers et al., 1990; Mandl et al., 1989; Henchal and Putnak, 1990; Putnak 1994; Rey et al., 1995).

Although many approaches to dengue virus vaccines have been pursued, there is no acceptable vaccine currently available. Until recently, the low titer of dengue virus grown in culture has made a killed vaccine impractical, and candidate live-attenuated dengue virus vaccine strains tested to date have proven unsatisfactory (see, e.g., Eckels et al, 1984; Bancroft et al, 1984; McKee et al, 1987), although live attenuated candidate vaccine strains continue to be developed and tested (Hoke et al, 1990; Bhamarapravati et al, 1987). The construction of several full-length infectious flavivirus clones (Rice *et al.*, 1989; Lai *et al.*, 1991; Sumiyoshi *et al.*, 1992) has facilitated studies aimed at identifying the determinants of virulence in flaviviruses (Bray and Lai, 1991; Chen *et al.*, 1995; Kawano *et al.*, 1993). However, these studies are in preliminary stages and little information on virulence has been obtained. A similar approach to vaccine development in the poliovirus system, while extremely informative, has taken years.

In the absence of effective live attenuated or killed flavivirus vaccines, a significant effort has been invested in the development of recombinant, flaviviral subunit or viral-vectored vaccines. Many of the vaccine efforts which use a recombinant DNA approach have focused on the E glycoprotein. This glycoprotein is a logical choice for a subunit vaccine as it is exposed on the surface of the virus and is believed to be responsible for eliciting protective immunity as monoclonal antibodies directed against purified flaviviral E proteins are neutralizing *in* vitro and some have been shown to confer passive protection *in* vivo (Henchal et al., 1985; Heinz et al., 1983; Mathews et al., 1984; Hawkes et al., 1988; Kimuro-Kuroda and Yasui, 1988).

Although the primary amino acid sequence of flaviviral E glycoproteins are variable (45-80% identity), all have twelve conserved cysteine residues, forming six disulfide bridges, and nearly superimposable hydrophilicity profiles suggesting that they probably have similar secondary and tertiary structures. Recently, the structure of a soluble fragment of the tick-borne encephalitis (TBE) virus envelope glycoprotein was solved at 2 Å resolution (Rey *et al.*, 1995). This analysis demonstrated that the envelope glycoprotein in its native form is a homodimer which presumably extends

parallel to the virion surface. This dimer is formed by an anti-parallel association of the two envelope glycoproteins stabilized by polar interactions along the central region of the dimer, and by non-polar interactions at either end (Figure 1). The dimer is slightly curved relative to the virion surface, perhaps conforming to the shape of the lipid envelope. The convex, external face contains the major immunogenic sites and the carbohydrate side chains. The carboxy terminus extends from the concave internal face down toward the membrane. Based upon sequence alignments and conservation of cysteine residues involved in disulfide bridges, the authors suggest that the TBE structure serves as a good model for all flavivirus envelopes. Therefore, recombinant soluble dengue E expressed as a dimer might induce a more potent antiviral response than monomeric E because it more closely resembles the natural envelope glycoprotein.

Recombinant flavivirus E glycoprotein has been expressed in several systems to date (See Putnak, 1994 for recent review). In general the systems have proven unsatisfactory for production of a cost-effective flavivirus vaccine due to limitations in antigen quality, quantity, or both. The following paragraphs highlight the major flavivirus vaccine efforts and summarize the results obtained to date.

Most efforts using *Escherichia coli* have yielded poor immunogen incapable of eliciting neutralizing antibodies in mice. This may reflect non-native conformation of flavivirus proteins expressed by bacteria and the necessity to process the viral proteins through the secretion pathway in order to achieve proper disulfide bond formation and glycosylation. Expression of dengue proteins using the eucaryotic yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* results in less than desirable quantities of immunogenic recombinant product obtained. The expression levels of dengue E achieved in these systems are well below that which would be required to produce a cost-effective flavivirus vaccine. (John Ivy et al., unpublished data)

Use of the baculovirus expression system for flavivirus subunit vaccine production has met with limited success (Reviewed in Putnak, Modern Vaccinology, 1994). In contrast to the high expression levels reported for various heterologous proteins in the baculovirus system, the levels of expression of flavivirus structural

proteins were quite low (*e.g.* 5-10μg DEN-2 E/10^6 cells; Deubel *et al.*, 1991), and reactivity against a panel of anti-flaviviral monoclonal antibodies (MAbs) indicated that many conformationally sensitive epitopes were not present (Deubel *et al.*, 1991). This suggests that folding of recombinant E produced in the baculovirus system may differ from the natural viral E protein. Furthermore, immunization with baculovirus-expressed recombinant envelope protein from DEN-1 (Putnak *et al.*, 1991), Japanese Encephalitis virus (McCown *et al.*, 1990), or Yellow Fever virus (Despres *et al.*, 1991) failed to elicit substantial titers of virus neutralizing antibodies or protection against viral challenge in mice.

Several reports have described vaccinia-flavivirus recombinants expressing envelope proteins as part of a polyprotein. The most consistently successful results in vaccinia expression of flaviviral proteins have been obtained co-expressing prM and E. Mice immunized with recombinant vaccinia expressing Japanese Encephalitis (JE) virus prM and E developed higher neutralizing antibody titers and survived higher challenge doses of virus (>10,000 LD_{50}; Konishi et al, 1992) than mice immunized with recombinant vaccinia virus expressing E alone (>10 LD_{50}; Mason et al, 1991). Similarly, mice immunized with a vaccinia-Yellow Fever (YF) virus recombinant expressing prM-E were protected from virus challenge at levels equivalent to that of the attenuated YFV-17D vaccine, while vaccinia-YF virus recombinants expressing E-NS1, C-prM-E-NS1, or NS1 failed to protect mice (Pincus et al, 1992). Vaccinia-DEN-1 recombinants expressing prM-E elicited neutralizing and hemagglutination inhibiting antibodies in mice, while recombinants expressing DEN-1 C-prM-E-NS1-NS2a-NS2b elicited no E-specific immune response (Fonseca et al, 1994).

Coordinate synthesis of prM and E appears to be important to obtain the native conformation of E. Expression of E in the absence of prM may result in a recombinant product that presents a different set of epitopes than those of the native virion (Konishi and Mason 1993; Heinz et al, 1994; Matsuura et al, 1989). Epitope mapping of the E expressed with prM suggests that the co-expressed protein more closely resembles the native virus. As prM and E appear to form heterodimers during viral maturation and E undergoes an acid pH-induced conformational change, Heinz et al

(1994) has suggested the association of prM and E is required to prevent irreversible pH-induced conformational changes during transit through the secretory pathway. However, it has been shown that carboxy-truncated forms of flavivirus E expressed in the absence of prM elicit protection from challenge (Men et al, 1991; Jan et al, 1993; Coller et al., in preparation), suggesting expression of E in the absence of prM can result in the display of protective epitopes.

Within the last ten years an alternative eucaryotic expression system which uses the *Drosophila melanogaster* Schneider 2 (S2) cell line has been developed and used to efficiently express the envelope glycoprotein of Human Immunodeficiency Virus (Ivey-Hoyle et al., 1991; Culp et al, 1991; van der Straten et al, 1989). We have applied this system to production of recombinant flavivirus subunit polypeptides and have found the system can easily produce 20-30 mg of recombinant protein per liter of medium (unpublished). The recombinant product we have focused most of our efforts on is a soluble form of flaviviral E, which is truncated at the carboxy-terminal end resulting in a polypeptide which represents approximately 80% of the full-length E molecule (amino acids 1-395; 80%E). We have expressed 80%E as a single open-reading frame with prM to enhance proper folding and secretion as described above. The expression levels achieved using this combination of expression system and recombinant DNA construct far exceed those achieved in other systems and does provide a cost-effective source of flaviviral antigen for vaccine production. In addition, we have demonstrated that the recombinant 80%E product secreted by these cells is capable of inducing neutralizing antibodies and protection in mice (Coller et al., in preparation.)

While the use of this combination of *Drosophila* S2 cells and prM80%E has allowed significant progress towards the production of an effective flavivirus vaccine, the ability of a small polypeptide, with limited antigenic complexity, to induce long term, protective immunity in a large, outbred population may be limited. Numerous studies have demonstrated that immunogenicity is directly related both to the size of the immunogen and to the antigenic complexity of the immunogen. Thus, in general, larger antigens make better immunogens. In addition, the structure of TBE envelope

protein was recently solved (Rey et al., 1995) and this analysis revealed that the native form of E protein found on the surface of the virion is a homodimer (Figure 1). Our recombinant flaviviral E protein discussed above is monomeric and therefore is not identical to the natural viral E protein. Thus, in an attempt to produce a recombinant flavivirus vaccine with enhanced immunogenicity we engineered several constructs designed to promote dimerization of the soluble 80%E which is so efficiently produced in the *Drosophila* cells. By enhancing dimerization we increase the potency of the vaccine by increasing the structural similarity to native, virally expressed E, as well as by increasing the size and antigenic complexity of the immunogen.

Several of the approaches we have adopted to enhance dimerization of soluble 80%E were originally developed for antibody engineering. Flexible peptide linkers have been used to link the variable heavy and variable light chain polypeptides in the engineering of single chain Fv's (scFv; Huston et al., 1988; Bird et al., 1988). These linkers, which are often repeated GlyGlyGlyGlySer (Gly4Ser) units, exhibit limited torsional constraints on the linked polypeptides, and therefore offer a reasonable option for covalently connecting the carboxy end of one 80%E moiety to the amino terminus of the second 80%E moiety. Based on the distance from the carboxy terminus of one subunit and the amino terminus of the other in the crystal structure of TBE 80%E dimers (F. Heinz, personal communication), we designed a peptide linker, made up predominantly of Gly4Ser repeats, to link the two 80%E molecules. The linker was designed to be slightly longer than the distance in the native molecule, in order to avoid torsional constraint on the association of the two 80%E moieties.

The second and third approaches to engineer 80%E dimers used strategies developed to engineer self-associating scFv miniantibodies. For homodimer mini-antibody expression, Pack et al. (1992; 1993) expressed the scFv as a fusion with a flexible linker hinge and one of two dimerization domains (Figure 2). One dimerization domain was a parallel coiled-coil helix of a leucine zipper from the yeast *GCN4* gene product (Landschulz et al., 1988; O'Shea et al., 1989). The other domain was two alpha helices spaced by a sharp turn that associate to form a homodimeric four-helix bundle (Ho and DeGrado, 1987). The hinge region used to link the

dimerization domains to the scFv was taken from an antibody hinge region to achieve maximum rotational flexibility. When these antibody-hinge-helix constructs were expressed in *E. coli*, homodimer miniantibodies spontaneously formed and could be extracted from the soluble protein fraction of cell lysates. These antibodies were indistinguishable from whole antibodies in functional affinity. To express secreted 80%E that can spontaneously dimerize, we have used these dimerization domains connected to the 80%E domains by a flexible Gly4Ser tether.

Disclosure of the Invention

The present invention discloses and claims vaccines containing, as an active ingredient, a secreted recombinantly produced dimeric form of truncated flaviviral envelope protein. The vaccines are capable of eliciting the production of neutralizing antibodies against flavivirus. In the illustrations below, the dimeric forms of truncated flaviviral envelope protein are formed 1) by directly linking two tandem copies of 80%E in a head to tail fashion via a flexible tether; 2) via the formation of a leucine zipper domain through the homodimeric association of two leucine zipper helices each fused to the carboxy terminus of an 80%E molecule; or 3) via the formation of a non-covalently associated four-helix bundle domain formed upon association of two helix-turn-helix moieties each attached to the carboxy terminus of an 80%E molecule. All products are expressed as a polyprotein including prM and the modified 80%E products are secreted from *Drosophila melanogaster* Schneider 2 cells using the human tissue plasminogen activator secretion signal sequence (tPA_L). Secreted products are generally more easily purified than those expressed intracellularly, facilitating vaccine production.

One embodiment of the present invention is directed to a vaccine for protection of a subject against infection by a *Flavivirus*. The vaccine contains, as active ingredient, the dimeric form of truncated envelope (E) protein of a flaviviral serotype, for example a dengue virus serotype. The dimeric truncated E is secreted as a recombinantly produced protein from eucaryotic cells. The vaccine may further contain portions of additional flaviviral serotype dimeric E proteins similarly

produced. A preferred embodiment of the present invention relates to a vaccine for the protection of a subject against infection by a dengue virus. The vaccine contains a therapeutically effective amount of a dimeric 80%E, where, the 80%E has been secreted as a recombinantly produced protein from eucaryotic cells, such as *Drosophila* cells. Further, the "80%E" refers in one instance to a polypeptide which spans from Met 1 to Gly 395 of the DEN-2 envelope protein. The sequences described in the present application represent the envelope protein from dengue type 2 virus; three additional distinct dengue serotypes have been recognized. Therefore, "80%E" also refers to the corresponding peptide region of the envelope protein of these serotypes, and to any naturally occurring variants, as well as corresponding peptide regions of the envelope (E) protein of other flaviviruses. For example, serotypes of dengue virus such as: DEN-1; DEN-2; DEN-3; and DEN-4, as well as serotypes of: Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF).

Other embodiments of the present invention are directed to three basic approaches for the construction of dimeric 80%E molecules. (See *infra*.) These include: linked 80%E dimer; 80%E ZipperI; 80%E ZipperII; and 80%E Bundle.

Still other embodiments of the present invention are directed to vaccines containing truncated envelope protein of dimeric 80%E of more than one serotype to form multivalent vaccines, (i.e., divalent, trivalent, tetravalent, etc.). For example, such embodiments of the present invention include: a vaccine containing a first dimeric 80%E product of one flaviviral serotype and a second dimeric 80%E product of a second flaviviral serotype, and a third dimeric 80%E product of a third flaviviral serotype and a fourth dimeric 80%E product of a fourth flaviviral serotype, as well as in combination with other dimeric 80%E, each of a separate serotype one from another, where all dimeric 80%Es have been secreted as recombinantly produced protein from eucaryotic cells, such as *Drosophila* cells. It is considered that the present invention clearly includes vaccines that are comprised of multivalent truncated envelope protein of dimeric 80%E, which embrace two, three, four or more serotypes. For example, these serotypes may include the following dengue virus serotypes:

DEN-1; DEN-2; DEN-3; and DEN-4, as well as other flavivirus serotypes of: Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF).

Additional embodiments of the present invention contemplate compositions of antibodies consisting essentially of antibodies generated in a mammalian subject administered an immunogenic amount of a vaccine containing dimeric 80%E as well as containing a first dimeric 80%E and a second dimeric 80%E, where both first and second dimeric 80%E have been secreted as recombinantly produced protein from eucaryotic cells, such as *Drosophila* cells. These vaccines could include multivalent truncated envelope protein of dimeric 80%E, which embrace two, three, four or more serotypes. These serotypes may include dengue virus serotypes: DEN-1; DEN-2; DEN-3; and DEN-4, as well as serotypes of: Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF).

Still other embodiments of the present invention are drawn to immortalized B cell lines, where the B cells have been generated in response to the administration to a mammalian subject of an immunogenic amount of a vaccine containing truncated envelope protein of dimeric 80%E of more than one serotype to form multivalent vaccines, (i.e., divalent, trivalent, tetravalent, etc.). For example, such embodiments of the present invention include: a vaccine containing a first dimeric 80%E product of one flaviviral serotype and a second dimeric 80%E product of a second flaviviral serotype, and a third dimeric 80%E product of a third flaviviral serotype and a fourth dimeric 80%E product of a fourth flaviviral serotype, as well as in combination with other dimeric 80%E, each of a separate serotype one from another, where all dimeric 80%Es have been secreted as recombinantly produced protein from eucaryotic cells, such as *Drosophila* cells. These vaccines could include multivalent truncated envelope protein of dimeric 80%E, which embrace two, three, four or more serotypes. These serotypes may include dengue virus serotypes: DEN-1; DEN-2; DEN-3; and DEN-4, as well as serotypes of: Japanese encephalitis virus (JE), Tick-borne

encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF).

Further embodiments of the present invention are drawn to monoclonal antibodies secreted by these immortalized B cell lines.

Still further embodiments of the present invention are drawn to methods to protect a subject against a *Flavivirus*. These methods include the step of administering in a suitable manner to a subject in need of such protection an effective amount of a vaccine containing dimeric 80%E on a schedule optimum for eliciting such a protective immunoreactive response.

Another embodiment of the present invention is directed to methods to utilize the dimeric form of truncated flavivirus envelope protein for diagnosis of infection in individuals at risk for the disease. The diagnostic contains, as active ingredient, the dimeric form of truncated envelope protein of a flavivirus serotype. The dimeric truncated E is secreted as a recombinantly produced protein from eucaryotic cells. The diagnostic may further contain portions of additional flavivirus serotype dimeric E proteins similarly produced.

A preferred embodiment of the present invention relates to an immunodiagnostic for the detection of a *Flavivirus*, where the immunodiagnostic contains, a dimeric 80%E that has been secreted as a recombinantly produced protein from eucaryotic cells, such as *Drosophila* cells. Specifically, a preferred embodiment of the present invention relates to an immunodiagnostic for the detection of a flavivirus. Embodiments of the present invention include immunodiagnostics for the detection of a *Flavivirus*, where the immunodiagnostic contains, dimeric 80%E of more than one serotype to form multivalent immunodiagnostics, (i.e., divalent, trivalent, tetravalent, etc.). For example, such embodiments of the present invention include: an immunodiagnostics containing a first dimeric 80%E product of one flaviviral serotype and a second dimeric 80%E product of a second flaviviral serotype, and a third dimeric 80%E product of a third flaviviral serotype and a fourth dimeric 80%E product of a fourth flaviviral serotype, as well as in combination with other dimeric 80%E, each of a separate serotype one from another, where all of the dimeric

80%Es have been secreted as recombinantly produced protein from eucaryotic cells, such as *Drosophila* cells.

The present invention includes the embodiments of immunodiagnostic kits for the detection of a *Flavivirus*, in a test subject. These immmunodiagnostic kits contain (a) dimeric 80%E, where the dimeric 80%E has been secreted as recombinantly produced protein from eucaryotic cells, such as *Drosophila* cells; (b) a suitable solid support phase coated with dimeric 80%E; and (c) labeled antibodies immunoreactive to antibodies from the test subject.

Other embodiments of the immunodiagnostic kits of the present invention include multivalent dimeric 80%E of more than one serotype to form multivalent immunodiagnostics, (i.e., divalent, trivalent, tetravalent, etc.). For example, such embodiments of the present invention include: an immunodiagnostics containing a first dimeric 80%E product of one flaviviral serotype and a second dimeric 80%E product of a second flaviviral serotype, and a third dimeric 80%E product of a third flaviviral serotype and a fourth dimeric 80%E product of a fourth flaviviral serotype, as well as in combination with other dimeric 80%E products, each of a separate serotype one from another, where all of the dimeric 80%E products have been secreted as recombinantly produced protein from eucaryotic cells, such as *Drosophila* cells.

Further embodiments of the present invention relate to compositions of matter, that include a vector host recombinant DNA expression system, containing: (a) a suitable eucaryotic host cell; (b) a suitable recombinant DNA expression vector; (c) DNA encoding dimeric 80%E, operably linked and under the control of a suitable promoter; and (d) where the DNA encoding dimeric 80%E is also operably linked to a secretory signal leader sequence. The present invention further includes embodiments of a vector host recombinant DNA system where the dimeric 80%E is selected from the group consisting of: linked 80%E dimer; 80%E ZipperI; 80%E ZipperII; and 80%E Bundle. A preferred embodiment of the present invention relates to a vector host recombinant DNA system where the eucaryotic host cell is a *Drosophila* cell.

Other compositions of matter embodied in the present invention include DNA sequences encoding dimeric 80%E, specifically including DNA sequences encoding: linked 80%E dimer; 80%E ZipperI; 80%E ZipperII; and 80%E Bundle.

Brief Description of the Drawings

Figure 1 is a drawing reproduced from Rey et al., showing the crystal structure of the envelope protein of Tick Borne Encephalitis virus.

Figure 2 is a drawing reproduced from Pack et al., which shows two the approaches used for miniantibody engineering applied to 80%E Dimer formation.

Figure 3 shows the partial nucleotide sequence and deduced amino acid sequence of the genome of DEN-2 PR159/S1 strain.

Figure 4 is a drawing illustrating the strategy used to generate cDNA encoding tandem copies of 80%E linked by a flexible tether.

Figure 5 is a drawing illustrating the cloning strategy used to introduce the carboxy-terminal portion of the first 80%E - linker - and amino terminal portion of the second 80%E molecule into a prM80%E cDNA clone.

Figure 6 is a drawing illustrating the cloning strategy used to introduce the linked tandem copies of 80%E into a *Drosophila* expression vector.

Figure 7 illustrates the cloning strategy used to introduce oligonucleotides encoding the leucine zipper and four-helix bundle dimerization domains into the linked 80%E dimer cDNA clone.

Figure 8 is a drawing illustrating the cloning strategy used to introduce the cDNA fragments encoding Linked 80%E Dimer, 80%E ZipperI, 80%E ZipperII, and 80%E Bundle into a *Drosophila* expression vector.

Figure 9 shows the SDS-PAGE analysis of the expressed dimeric 80%E products secreted from transfected S2 cells.

Figure 10 demonstrates the glycosylation of the secreted dimeric 80%E products by SDS-PAGE analysis of endoglycosidase-digested 80%E dimers.

Figure 11 demonstrates the application of immunoaffinity techniques to purification of the secreted dimeric 80%E products.

Modes of Carrying Out the Invention

The invention provides, for the first time, a subunit vaccine with increased immunogenicity that can be efficiently produced and secreted using a recombinant expression system and that is effective in inducing a strong virus neutralizing response to flaviviruses. Although many attempts have been made to obtain such a subunit vaccine, previous studies were plagued with either low expression levels of an effective immunogen or efficient production of an ineffective vaccine candidate. The present applicants have found that recombinantly-engineered, dimeric forms of a carboxy-terminally truncated flaviviral envelope protein, corresponding to amino acids 1-395, are efficiently secreted by certain convenient eucaryotic recombinant hosts, in a form that permits processing to mimic the native conformation of the protein. The efficient secretion of the proteins into the culture medium facilitates purification. Furthermore, the secreted forms are able, especially when administered in the presence of adjuvant, to raise high titer virus neutralizing antibodies in animals. Thus, these proteins represents a useful component of a vaccine for protecting subjects against flaviviral infection.

As used herein, "80%E" refers in one instance to a polypeptide which spans from Met 1 to Gly 395 of the DEN-2 envelope protein. The sequences described in the present application represent the envelope protein from dengue type 2 virus; three additional distinct dengue serotypes have been recognized. Therefore, "80%E" also refers to the corresponding peptide region of the envelope protein of these serotypes, and to any naturally occurring variants, as well as corresponding peptide regions of the envelope (E) protein of other flaviviruses. For example, serotypes of dengue virus such as: DEN-1; DEN-2; DEN-3; and DEN-4, as well as serotypes of: Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF). The modifications made to the 80%E products by addition of carboxy-terminal sequences encoding flexible linkers, leucine zipper domains, or four helix bundle domains, designed to enhance the dimerization of the 80%E molecules, are described in detail below. All of these

dimeric 80%E proteins are produced from vectors containing the DNA encoding the flavivirus prM as a fusion with mature proteins resulting in secretion of the processed, mature dimeric 80%E proteins from which the prM protein has been removed.

Three basic approaches have been used to construct dimeric 80%E molecules. The first approach involves using tandem copies of 80%E covalently attached to each other by a flexible linker. As used herein, "Linked 80%E Dimer" refers in one instance to a polypeptide which encodes DEN-2 80%E - GGGSGGGGSGGGTGGGSGGGSGGGG - DEN-2 80%E. The stretch of amino acids covalently linking the two copies of DEN2 80%E is designed to serve as a flexible tether allowing the two 80%E molecules to associate in native head-to-tail dimeric orientation while maintaining their covalent attachment to each other. The sequences described in the present application represent the envelope protein from dengue type 2 virus; three additional distinct dengue serotypes have been recognized. Therefore, "Linked 80%E Dimer" also refers to the corresponding peptide region of the envelope protein of these serotypes, and to any naturally occurring variants, as well as corresponding peptide regions of the envelope (E) protein of other flaviviruses. For example, serotypes of dengue virus such as: DEN-1; DEN-2; DEN-3; and DEN-4, as well as serotypes of: Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF).

It would be readily apparent to one of ordinary skill in the art to select other linker sequences as well. The present invention is not limited to the specific disclosed linkers, but, to any amino acid sequence that would enable the two 80%E molecules to associate in native head to tail dimeric orientation while maintaining their covalent attachment to each other.

The second approach involves addition of a carboxy-terminal leucine zipper domain to monomeric 80%E to enhance dimerization between two 80%E-leucine zipper molecules. Two versions of this approach have been adopted. One version includes a disulfide bond linking the leucine zipper domains resulting in a covalently linked dimer product, while the other is based on the non-covalent association of the

leucine zipper domains. As used herein "80%E ZipperI" refers in one instance to a polypeptide which encodes DEN-2 80%E - GGGSGGGGSGGGTGGGSGGGSP-RMKQLEDKVEELLSKNYHLENEVARLKKLVGER. The first 22 amino acids extending after the carboxy terminus of 80%E serve as flexible tether between 80%E and the adjacent leucine zipper domain. The leucine zipper domain is designed to dimerize with the identical sequence from another 80%E Zipper molecule. The formation of a non-covalently linked leucine zipper will enhance the dimerization of the 80%E molecules, which may associate in native head to tail conformation by virtue of the flexible linker connecting the 80%E molecules with the leucine zipper domain. The sequences described in the present application represent the envelope protein from dengue type 2 virus; three additional distinct dengue serotypes have been recognized. Therefore, "80%E ZipperI" also refers to the corresponding peptide region of the envelope protein of these serotypes, and to any naturally occurring variants, as well as corresponding peptide regions of the envelope (E) protein of other flaviviruses. For example, serotypes of dengue virus such as: DEN-1; DEN-2; DEN-3; and DEN-4, as well as serotypes of: Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF).

It would be readily apparent to one of ordinary skill in the art to select other leucine zipper sequences as well. The present invention is not limited to the specific disclosed leucine zipper sequences, but to any amino acid sequences that would enable the dimerization between identical sequences from another 80%E Zipper molecule.

As used herein "80%E ZipperII" refers in one instance to a polypeptide which encodes DEN-2 80%E - GGGSGGGGSGGGTGGGSGGGSP-RMKQLEDKVEELLSKNYHLENEVARLKKLVGERGGCGG. The first 22 amino acids extending after the carboxy terminus of 80%E serve as flexible tether between 80%E and the adjacent leucine zipper domain. The leucine zipper domain is designed to dimerize with the identical sequence from another 80%E Zipper molecule. The leucine zipper domain of 80%E ZipperII ends in a GGCGG sequence which facilitates

disulfide bond formation between the two leucine zipper helices. Thus, once the leucine zipper dimerizes, a disulfide bond forms between the two ends, resulting in a covalently linked dimer product. The formation of a covalently linked leucine zipper will enhance the dimerization of the 80%E molecules, which may associate in native head to tail conformation by virtue of the flexible linker connecting the 80%E molecules with the leucine zipper domain. The sequences described in the present application represent the envelope protein from dengue type 2 virus; three additional distinct dengue serotypes have been recognized. Therefore, "80%E ZipperII" also refers to the corresponding peptide region of the envelope protein of these serotypes, and to any naturally occurring variants, as well as corresponding peptide regions of the envelope (E) protein of other flaviviruses. For example, serotypes of dengue virus such as: DEN-1; DEN-2; DEN-3; and DEN-4, as well as serotypes of: Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF).

It would be readily apparent to one of ordinary skill in the art to select other leucine zipper sequences as well. The present invention is not limited to the specific disclosed leucine sequences, but to any amino acid sequences that would permit the dimerizeration with an identical sequence from another 80%E Zipper molecule. Further, the ordinary skilled artisan would readily be able to determine other sequences that would facilitate disulfide bond formation between the two leucine zipper helices.

The final approach used to enhance dimerization of 80%E is the addition of a helix-turn-helix domain to the carboxy terminal end of 80%E. The helix-turn-helix domain from one modified 80%E molecule will associate with that of another to form a dimeric four-helix bundle domain. As used herein "80%E Bundle" refers in one instance to a polypeptide which encodes DEN-2 80%E-GGGSGGGGSGGGTGGGSGGGSP-GELEELLKHLKELLKG-PRK-GELEELLKHLKELLKGEF. The first 22 amino acids extending after the carboxy terminus of 80%E serve as flexible tether between the 80%E domain and the helix-turn-helix domain which follows. The formation of a non-covalently associated four-

helix bundle domain will enhance the dimerization of the 80%E molecules which may associate in the native head to tail conformation by virtue of the flexible linkers connecting 80%E to the helix bundle. The sequences described in the present application represent the envelope protein from dengue type 2 virus; three additional distinct dengue serotypes have been recognized. Therefore, "80%E Bundle" also refers to the corresponding peptide region of the envelope protein of these serotypes, and to any naturally occurring variants, as well as corresponding peptide regions of the envelope (E) protein of other flaviviruses. For example, serotypes of dengue virus such as: DEN-1; DEN-2; DEN-3; and DEN-4, as well as serotypes of: Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), West Nile virus (WN), and the family prototype, yellow fever virus (YF).

It would be readily apparent to one of ordinary skill of the art to select other amino acid sequences that would form the flexible tether extending after the carboxy terminal of the 80%E and also comprising a helix-turn-helix domain. The present invention is not limited to the specific disclosed helix-turn-helix domains, but to any amino acid sequences that would enable the dimerization of one modified 80%E molecule through a non-covalent association with a second modified 80%E molecule. Further, the ordinary skilled artisan would readily be able to determine other sequences that would facilitate such non-covalent association of helices.

Recombinant techniques provide the most practical approach for practical large-scale production of these subunits for vaccine and diagnostic purposes. However, to be efficacious these proteins must undergo correct processing and assume a conformation similar to that of native flaviviral envelope protein. In order to achieve this, the recombinant production must be conducted in eucaryotic cells, preferably *Drosophila melanogaster* cells. Other eucaryotic cells including yeast, mammalian cells such as Chinese hamster ovary cells, or additional types of insect cells may also be used. However, to make a cost-effective vaccine feasible, the dimeric 80%E products must be efficiently secreted with correct processing and folding.

It has been found, as demonstrated herein below, that particularly efficient secretion of biologically active mature protein is most easily achieved using the *Drosophila melanogaster* Schneider-2 cell line. The expression of the dimeric products is driven by an efficient insect cell promoter (*Drosophila* metallothionein promoter) and secretion is targeted using a eucaryotic secretion leader (human tissue plasminogen activator secretion leader) as well as the flaviviral prM protein which contains the secretion signal for E. Other promoters and secretion leaders can also be used. In general, the invention includes expression systems that are operable in eucaryotic cells and which result in the secretion of dimeric truncated flaviviral envelope proteins into the medium. Thus, useful in the invention are cells and cell cultures which contain expression systems resulting in the production and secretion of mature dimeric truncated flaviviral envelope proteins.

The properly processed dimeric truncated E proteins are recovered from the cell culture medium, purified, and formulated into vaccines. Purification and vaccine formulation employ standard techniques and are matters of routine optimization. Suitable formulations are found, for example, in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Company, Easton, PA. In particular, formulations will include an adjuvant, such as alum or other effective adjuvant. Alternatively, the active ingredient and the adjuvant may be coadministered in separate formulations.

The active vaccines of the invention can be used alone or in combination with other active vaccines such as those containing attenuated or killed forms of the virus, or those containing other active subunits to the extent that they become available. The vaccines may contain only one subunit as an active ingredient, or additional isolated active components may be added. Corresponding or different subunits from one or several serotypes may be included in a particular formulation.

To immunize subjects against flaviviral infection, the vaccines containing therapeutically effective amounts of the subunit are administered to the subject in conventional immunization protocols involving, usually, multiple administrations of the vaccine. Administration is typically by injection, typically intramuscular or

subcutaneous injection; however, other systemic modes of administration may also be employed. Less frequently used, transmucosal and transdermal formulations are included within the scope of the invention as are effective means of oral administration. The efficacy of these formulations is a function of the development of formulation technology rather than the contribution of the present invention.

In addition to use in vaccines, the recombinant dimeric truncated E proteins of the invention may be used as analytical reagents in assessing the presence or absence of anti-flaviviral antibodies in samples. Such uses include, but are not limited to, diagnosis of infection with any flavivirus, such as dengue, monitoring the response to flaviviral infection, or use of immunoassays as part of standard laboratory procedures in the study of the progress of antibody formation or in epitope mapping and the like. The antigens are employed in standard immunoassay formats with standard detection systems such as enzyme-based, fluorescence-based, or isotope-based detection systems. Preferably, the antigen is used coupled to solid support or in sandwich assays, but a multiplicity of protocols is possible and standard in the art.

Thus, the secreted dimeric proteins, linked 80%E dimer, 80%E ZipperI, 80%E ZipperII, or 80%E Bundle, may be adsorbed onto solid support and the support then treated with a sample to be tested for the presence of anti-flaviviral antibodies. Unbound sample is removed, and any bound antibodies are detected using standard detection systems, for example, by treating the support with an anti-species antibody coupled to a detection reagent, for example horseradish peroxidase (HRP), with the species specificity of the antibody determined by the sample being tested. The presence of the HRP-conjugated antispecies antibody is then detected by supplying a suitable chromogenic substrate. In addition, the dimeric proteins may be used to detect the presence or absence of antibodies of various isotypes, including IgG and IgM isotypes by simply altering the specificity of the detecting antibodies. This may be particularly significant as IgM antibodies to flavivirus are considered diagnostic of a primary flaviviral infection. Alternatively, the anti-subunit or anti-flaviviral antibody may be adsorbed to the solid support and detected by treating the solid support with

the recombinant dimeric proteins, either directly labeled, or labeled with an additional antibody in a sandwich-type assay.

In another embodiment, this invention relates to diagnostic kits comprising an antigen affixed to a solid support phase and an immunological detection system. The antigen of this invention is a secreted dimeric product used in conjunction with an immunological detection system. The antigen includes the recombinant dimeric truncated E protein in the form of a linked 80%E dimer or an 80%E ZipperI or an 80%E ZipperII or an 80%E bundle. The solid support phase of this invention relates to any of those found in the art, including microtiter plates. The detection system of this invention relates to any of those found in the art including antihuman antibodies conjugated with a detectable enzyme label.

In the examples below, the expression, secretion, processing, and immunogenicity of the secreted dimeric proteins, linked 80%E dimer, 80%E ZipperI, 80%E ZipperII, and 80%E Bundle are demonstrated. The products are recombinantly produced as modified prM-80%E fusions which are efficiently processed to remove the prM portion and secreted from *Drosophila* cells. The secreted dimeric 80%E products are secreted at high levels, up to 10 μg/ml in unselected cells, and they display a complex pattern of glycosylation typical of mammalian and insect cell expression systems. Furthermore, based upon reactivity with conformationally sensitive monoclonal antibodies, the secreted dimeric 80%E products have native-like conformation and immunization of mice with dimeric 80%E, either crude or purified, induces a potent virus-neutralizing immune response.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Construction of expression vector pMttD2prM2X80E for secretion of Linked 80%E Dimer

DEN-2 strain PR159/S1 served as the source for all DEN-2 genes used in the invention. This strain has a small plaque, temperature-sensitive phenotype and differs

from wild-type DEN-2 PR159 strain at only one amino acid in the prM and E coding regions. A cDNA clone, pC8 (Hahn et al, 1988), derived from DEN-2 strain PR159/S1 was used as starting material for generation of the subclones described below. The sequence of the clone has been previously published (Hahn et al., 1988), however, complete sequencing of the pC8 clone, as well as subclones derived from pC8, in our laboratory has identified a number of discrepancies with the published sequence. The complete nucleotide sequence and deduced amino acid sequence of the cDNA encoding the viral capsid, prM, E, and NS1 genes for PR159/S1 is included in Figure 3. Shown in bold (and indicated with a *) at nucleotides 103, 1940, 1991, and 2025 are corrections to the Hahn published sequence.

The pC8 cDNA clone was used to generate several subclones critical for the construction of the dimeric 80%E clones included in this invention. The first subclone encodes amino acids 1-395 of E (80%E). The primers D2E937p and D2E2121m, shown below, were used to amplify the cDNA fragment extending from nucleotide 937 to 2121 and corresponding to 80%E. These primers include convenient restriction sites for cloning and the D2E2121m primer includes two stop codons after the 395th codon of E. The sequence of the primers is listed below with dengue sequence listed in uppercase letters and non-dengue sequences listed in lowercase letters.

```
                    Bgl II
D2E937p  5' - cttctagatctcgagtacccgggacc ATG CGC TGC ATA GGA ATA TC -
3'
                 XbaI   XhoI    SmaI     Met Arg Cys Ile Gly Ile Ser

                         Sal I
D2E2121m  5' - gctctagagtcga cta tta TCC TTT CTT GAA CCA G - 3'
                  XbaI       END END Gly Lys Lys Phe Trp
```

The amplified 80%E cDNA fragment was digested with XbaI and cloned into the NheI site of pBR322 to obtain the plasmid p29D280E. The complete nucleotide sequence of the clone was determined and a single, silent, PCR-induced mutation at nucleotide 2001 (AAC/Asn to AAT/Asn) was identified.

The portion of the genome that encodes prM and E was subcloned from pC8 using the Polymerase Chain Reaction (PCR). Oligonucleotide primers were designed to amplify the region of the genome, nucleotides 439 to 2421, corresponding to amino acids 1-166 of prM and 1-495 of E with convenient restriction sites engineered into the primers to facilitate cloning. In addition the primer used to amplify the amino terminus of the prM-E polyprotein includes a methionine codon (ATG) immediately preceding the first codon (phenylalanine) of the prM coding sequence. The sequence of the primers is listed below with dengue sequence listed in uppercase letters and non-dengue sequences listed in lowercase letters.

```
                    Bgl II
D2prM439p 5' - attctagatctcgagtacccgggacc atg TTT CAT CTG ACC ACA CGC
-3'
                  XbaI  XhoI    SmaI     Met Phe His Leu Thr Thr Arg

                         Sal I
D2E2421m  5' - tctctagagtcga cta tta GGC CTG CAC CAT AAC TCC - 3'
                  XbaI       END END Ala Gln Val Met Val Gly
```

The PCR-generated prM100%E cDNA fragment was digested with the restriction endonuclease XbaI and ligated into the XbaI site of pBluescript SK+ (Stratagene, La Jolla, CA) to obtain the plasmid p29prME13. DNA sequence analysis of the PCR-generated cDNA clone identified two PCR-induced nucleotide differences between pC8 and p29prME13 in the prM-80%E coding region. The first mutation involves a T to C transition at nucleotide 1255 which is silent, and the second change involves an A to G transition at nucleotide 1117 which results in the conservative amino acid substitution of a valine for an isoleucine at position 61 of E. This mutation was repaired by replacing an AflII fragment containing the mutation with the corresponding AflII fragment from pC8 encoding the correct sequence.

To generate a cDNA subclone representing prM80%E, a 794 bp BamHI-SalI fragment, representing the carboxy-terminal end of E, was removed from p29prME13 and replaced with the 431 bp BamHI-SalI fragment from p29D280E, encoding the carboxy-terminal end of 80%E. The BamHI site is a naturally occurring site within

the envelope cDNA, and the SalI site is an engineered site that immediately follows the stop codons encoded by the PCR primers. The resulting truncated cDNA clone, pBsD2prM80E, was confirmed by restriction digestion and DNA sequence analysis to encode amino acids 1 through 166 of prM and 1 through 395 of envelope.

To engineer the Linked 80%E Dimer, cDNA encoding 80%E was PCR amplified in two "halves" from pC8 using primer/adapters that include the flexible linker and a KpnI restriction endonuclease site to facilitate ligation of the two halves. One half, designated PCR 1, encoded the carboxy terminus of the flexible linker and the amino terminus of 80%E. The other half, designated PCR 2 encoded the carboxy terminus of 80%E and the amino terminus of the flexible linker. The nucleotide sequences of the primers used to amplify the PCR 1 and PCR 2 cDNAs are listed below. In each case, the cDNA fragments spanned a naturally occurring, unique BamHI site within the 80%E coding region. The strategy for generating and cloning the fragments is outlined in Figure 4. The PCR products were digested with PstI and BamHI and cloned individually into pUC plasmid vectors cut with the same two enzymes, resulting in plasmids pUC18PCR1 and pUC13PCR2 which were confirmed by DNA sequence analysis. The fragment encoding the amino terminus of 80%E was released from the pUC18PCR1 subclone by digestion with KpnI and cloned into pUC13PCR2 linearized with KpnI to generate the clone pUC13PCR2+1 which encodes the carboxy terminus of 80%E - flexible linker - amino terminus of 80%E.

The primers used to generate cDNA fragment PCR1 were:

```
                PstI  KpnI
DI80E-2N 5'
AGTCCTGCAGGTACCGGTGGTGGTGGTTCTGGTGGTGGTTCTGGTGGTGGTATGCGTTGCATA
a.a. sequence          T  G  G  G  G  S  G  G  G  S  G  G  G  M  R  C
I

            GGAATATCAAATAGG
             G  I  S  N  R

D2E2007M  5' CTATGATGATGTAGCTGTCTCC
a.a. sequence  I  I  I  Y  S  D  G
```

The primers used to generate cDNA product PCR 2 were:

```
                        PstI  KpnI
DI80E-1C  5'
GCTCAGCTGCAGGTACCACCACCAGAACCACCACCACCAGAACCACCACCACCTTTCTT
a.a. sequence                G  G  G  S  G  G  G  G  S  G  G  G  G  K

K

              GAACCAGTCCAGC
               F  W  D  L

D2E1642P  5'  GACACTGGTCACCTT
a.a. sequence   T  L  V  T  F
```

To generate the sequence encoding prM plus the tandemly linked copies of 80%E, the cDNA fragment encoding carboxy terminus 80%E - flexible linker - amino terminus 80%E was released from the pUC13PCR2+1 clone by digestion with BamHI. This BamHI fragment was then ligated into pBsD2prM80E digested with BamHI to yield pBsD2prM2X80E (Figure 5).

To facilitate manipulations of the linked 80%E dimer expression plasmid, we modified the *Drosophila melanogaster* expression vector pMttbns (SmithKline Beecham). A XhoI site at nucleotide 885 was deleted by removing a 19 base pair BamHI fragment containing the XhoI site. The resulting pMtt-Xho plasmid contained a unique XhoI site at nucleotide 730 which precedes the SV40 polyadenylation signal and is useful for introducing genes for expression studies. Plasmid pMtt-Xho was further modified to delete a KpnI site just upstream of the metallothionein promoter so that upon introduction of the linked 80%E dimer sequences, the KpnI site in the linker will be unique in the clone. To accomplish this, the pMtt-Xho plasmid was digested with the restriction endonuclease Acc65I. This enzyme has the same recognition sequence as KpnI but upon digestion results in a 5' overhang which can be made flush upon incubation with Klenow fragment of DNA polymerase I and deoxyribonucleotides. Thus digestion of pMtt-Xho with Acc65I followed with Klenow treatment and ligation resulted in a plasmid, pMtt-HBG, which lacks the KpnI site (Figure 6).

To introduce the linked 80%E dimer into the pMtt-HBG expression plasmid, pBsD2prM2X80E was digested with BglII and SalI to release the prM - 80%E - linker

- 80%E encoding fragment. This fragment was ligated into pMtt-HBG digested with BglII/SalI (Figure 6). DNA sequence analysis of the resulting plasmid, pMttHBGD2prM2X80E, confirmed that the clone contained the entire prM2X80E coding sequence but lacked the SV40 polyadenylation signal. This clone is useful for introducing the oligonucleotides encoding the leucine zipper and four-helix bundle domains (Examples 2, 3, and 4) but is not useful for expression studies, as no poly A tail is associated with low expression levels. To restore the poly adenylation signal, the BglII/SalI fragment containing prM2X80E was removed from the pMttHBGD2prM2X80E clone and ligated into the pMtt-Xho plasmid digested with BglII and XhoI (Figure 8). The resulting plasmid, pMttD2prM2X80E, was used for transfection of *Drosophila* cells and expression studies.

Example 2

Construction of expression vector pMttD2prM80EZipperI for secretion of non-covalently linked 80%E ZipperI

The plasmid pMttHBGD2prM2X80E was used as backbone for the introduction of oligonucleotides encoding one half of the flexible Gly4Ser linker and the leucine zipper coiled coil helix. As illustrated in Figure 7, this plasmid was digested with KpnI and SalI to remove a fragment containing the carboxy-terminal half the flexible linker and the second copy of 80%E. Four overlapping oligonucleotides, coding for the carboxy-terminal half of the linker and leucine zipper helix were annealed to each other, generating a KpnI site at the 5' end and SalI site at the 3' end. The nucleotide and encoded amino acid sequence of the overlapping oligonucleotides are listed below. The annealed oligos were ligated into the KpnI/SalI digested vector to generate the expression plasmid, pMttHBGprM80EZipI. The identity of the pMttHBGprM80EZipI clone was confirmed by restriction digestion and limited sequence analysis.

As described above however, the pMttHBGD2prM2X80E used as backbone for this construct lacks the SV40 polyadenylation sequence. Therefore, the BglII/SalI fragment from pMttHBGprM80EZipI, encoding prM80%E ZipperI, was removed

from the pMttHBGprM80EZipI plasmid and cloned into the BglII/XhoI digested pMtt-Xho vector to restore the downstream polyadenylation signal (Figure 8). The resulting plasmid, pMttD2prM80EZipI, was confirmed by restriction digestion and sequence analysis and used to transfect *Drosophila* cells for expression studies.

Oligonucleotide Sequences:

```
5'
GTACCGGCGGTGGCTCCGGCGGTGGCTCCCCCCGCATGAAGCAGCTGGAGGACAAGGTGGAGGAGCTGCT
3'
GCCGCCACCGAGGCCGCCACCGAGGGGGGCGTACTTCGTCGACCTCCTGTTCCACCTCCTCGACGA

a.a.  T  G  G  G  S  G  G  G  S  P  R  M  K  Q  L  E  D  K  V  E  E  L
L

GTCCAAGAACTACCACCTGGAGAACGAGGTGGCCCGCCTGAAGAAGCTGGTGGGCGAGCGCTAATAGG
3'
CAGGTTCTTCATGGTGGACCTCTTGCTCCACCGGGCGGACTTCTTCGACCACCCGCTCGCGATTATCCAGCT
5'

  S  K  N  Y  H  L  E  N  E  V  A  R  L  K  K  L  V  G  E  R
```

Example 3

Construction of expression vector pMttD2prM80EZipperII

for secretion of covalently linked 80%E ZipperII

The plasmid pMttHBGD2prM2X80E was used as backbone for the introduction of oligonucleotides encoding one half of the flexible Gly4Ser linker and the leucine zipper coiled coil helix with a cysteine residue close to the carboxy terminus. As illustrated in Figure 7, this plasmid was digested with KpnI and SalI to remove a fragment containing carboxy-terminal half of the linker and the second copy of 80%E. Four overlapping oligonucleotides, coding for the carboxy-terminal half of the linker and cysteine-containing leucine zipper helix were annealed to each other, generating a KpnI site at the 5' end and SalI site at the 3' end. The nucleotide and encoded amino acid sequences of the overlapping oligonucleotides are listed below. The annealed oligos were ligated into the KpnI/SalI digested vector to generate the expression plasmid, pMttHBGprM80EZipII. The identity of the

pMttHBGprM80EZipII clone was confirmed by restriction digestion and limited sequence analysis.

As described above however, the pMttHBGD2prM2X80E used as backbone for this construct lacks the SV40 polyadenylation sequence. Therefore, the BglII/SalI fragment from pMttHBGprM80EZipII, encoding prM80%E ZipperII, was removed from the pMttHBGprM80EZipII plasmid and cloned into the BglII/XhoI digested pMtt-Xho vector to restore the downstream polyadenylation signal (Figure 8). The resulting plasmid, pMttD2prM80EZipII, was confirmed by restriction digestion and sequence analysis and used to transfect *Drosophila* cells for expression studies.

Oligonucleotide Sequences:

```
5'
GTACCGGCGGTGGCTCCGGCGGTGGCTCCCCCCGCATGAAGCAGCTGGAGGACAAGGTGGAGGAGCTGCT
3'
GCCGCCACCGAGGCCGCCACCGAGGGGGGCGTACTTCGTCGACCTCCTGTTCCACCTCCTCGACGA

a.a.   T  G  G  G  S  G  G  G  S  P  R  M  K  Q  L  E  D  K  V  E  E  L
L

GTCCAAGAACTACCACCTGGAGAACGAGGTGGCCCGCCTGAAGAAGCTGGTGGGCGAGCGCGGCGGTTGCGG
CGG
CAGGTTCTTCATGGTGGACCTCTTGCTCCACCGGGCGGACTTCTTCGACCACCCGCTCGCGCCGCCAACGCC
GCC

  S  K  N  Y  H  L  E  N  E  V  A  R  L  K  K  L  V  G  E  R  G  G  C  G
G

TTAATAGG 3'
AATTATCCAGCT 5'
```

Example 4

Construction of expression vector pMttD2prM80EBundle for secretion of non-covalently linked 80%E Bundle

The plasmid pMttHBGD2prM2X80E was used as backbone for the introduction of oligonucleotides encoding one half of the flexible Gly4Ser linker and the helix-turn-helix domain. As illustrated in Figure 7, this plasmid was digested with KpnI and SalI to remove a fragment containing the carboxy-terminal half of the linker and the second copy of 80%E. Four overlapping oligonucleotides, coding for the

carboxy-terminal half of the linker and helix-turn-helix domain were annealed to each other, generating a KpnI site at the 5' end and SalI site at the 3' end. The nucleotide and encoded amino acid sequences of the overlapping oligonucleotides are listed below. The annealed oligos were ligated into the KpnI/SalI digested vector to generate the expression plasmid, pMttHBGprM80EBundle. The identity of the pMttHBGprM80EBundle clone was confirmed by restriction digestion and limited sequence analysis.

As described above however, the pMttHBGD2prM2X80E used as backbone for this construct lacks the SV40 polyadenylation sequence. Therefore, the BglII/SalI fragment from pMttHBGprM80EBundle, encoding prM80%E Bundle, was removed from the pMttHBGprM80EBundle plasmid and cloned into the BglII/XhoI digested pMtt-Xho vector to restore the downstream polyadenylation signal (Figure 8). The resulting plasmid, pMttD2prM80EBundle, was confirmed by restriction digestion and sequence analysis and used to transfect *Drosophila* cells for expression studies.

Oligonucleotide Sequences:

```
5'
GTACCGGCGGTGGCTCCGGCGGTGGCTCCCCCGGCGAGCTGGAGGAGCTGCTGAAGCACCTGAAGGAG
3'
GCCGCCACCGAGGCCGCCACCGAGGGGGCCGCTCGACCTCCTCGACGACTTCGTGGACTTCCTC

a.a.   T  G  G  G  S  G  G  G  S  P  G  E  L  E  E  L  L  K  H  L  K  E

CTGCTGAAGGGCCCCCGCAAGGGCGAGCTGGAGGAGCTGCTGAAGCACCTGAAGGAGCTGCTGAAGGGCGAG
GACGACTTCCCGGGGGCGTTCCCGCTCGACCTCCTCGACGACTTCGTGGACTTCCTCGACGACTTCCCGCTC

 L  L  K  G  P  R  K  G  E  L  E  E  L  L  K  H  L  K  E  L  L  K  G  E

TTCTAATAGG 3'
AAGATTATCCAGCT 5'
 F
```

Example 5

Expression and Secretion of Linked 80%E Dimer, 80%E ZipperI, 80%E ZipperII, and 80%E Bundle from *Drosophila melanogaster* S2 cells

Drosophila melanogaster Schneider-2 cells (S2; ATCC, Rockville, MD) were cotransfected with each of the expression plasmids described in detail above

(pMttD2prM2X80Ef, pMttD2prM80EZipperI, pMttD2prM80EZipperII, or pMttD2prM80EBundle) and the selection plasmid, pCoHygro, at a weight ratio of 20:1 using the calcium phosphate coprecipitation method (Wigler et al., 1979; Gibco BRL, Grand Island, NY). The pCoHygro selection plasmid (van der Straten et al., 1989; SmithKline Beecham) encodes the *E.* coli hygromycin B phosphotransferase gene under thr transcriptional control of the *D. melanogaster* copia transposable element long terminal repeat and confers resistance to hygromycin B. Transfectants were selected for outgrowth in Schneider's medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 300 μg/ml hygromycin B (Boerhinger Mannheim). Following significant outgrowth, transfectants were plated at a cell density of 2 X 10^6 cell/ml in serum-free IPL-41 medium supplemented with lipids, yeastolate, and Pluronic F68 (Gibco BRL) and induced with 200 μM $CuSO_4$. The media were harvested after 7 days of induction.

Proteins secreted into the culture medium were separated by SDS-PAGE, and analyzed by Coomassie blue staining and immunoprobing of Western blots with a polyclonal anti-DEN2 domain B (domain B corresponds to amino acids 296-395 of E). Under non-reducing conditions the expected sizes for Linked 80%E Dimer, 80%E ZipperI, 80%E ZipperII, and 80%E Bundle are 89.1 kD, 49.2 kD, 99.5 kD, and 49.5 kD respectively. An immunoreactive band of appropriate molecular weight was detected in culture medium from all four constructs (Figure 9A). This analysis confirms that 80%E ZipperII, which was designed with cysteine residues near the carboxy terminal end of the leucine zipper alpha helices to facilitate disulfide bond formation, is covalently dimerized by the disulfide bond. This is in contrast to the non-covalently associated 80%E ZipperI and 80%E Bundle products which migrate as monomers under denaturing but non-reducing conditions. Coomassie blue staining of the crude media reveals a unique band which is plainly visible in the 80%E ZipperI, 80%E ZipperII, and 80%E Bundle lanes (Figure 9B). Comigrating bands of similar size make visualization of the Linked 80%E Dimer band more difficult. Based upon staining of protein standards we estimate the concentrations of the dimeric proteins to be between 5 and 15 μg/ml depending on the construct and the growth conditions.

Thus all four dimeric 80%E proteins are expressed to high levels and efficiently secreted from transfected Drosophila S2 cultures.

Example 6

Secreted Dimeric 80%E Products are Glycosylated

Native dengue viral E is a glycoprotein displaying a complex pattern of glycosylation typical of mammalian- and insect cell-expressed proteins. Additional analyses of the secreted recombinant dimeric 80%E products demonstrated that all four of the products are glycosylated. Crude media containing Linked 80%E Dimer, 80%E ZipperI, or 80%E Bundle or purified 80%E ZipperII were denatured upon heat treatment with SDS and 2-mercaptoethanol prior to digestion with endoglycosidase H (EndoH) or peptide:N-glycosidase F (PNGase F). Digested and undigested control preparations were separated on SDS-PAGE gels and analyzed by Coomassie blue staining or Western blot analysis. Western blots probed with polyclonal anti-DEN2 hyperimmune mouse ascites fluid (HMAF) demonstrate that all dimeric products are resistant to EndoH digestion but sensitive to PNGase F digestion consistent with a complex pattern of glycosylation (Figure 10). Thus, the glycosylation pattern of all four recombinant dimeric 80%E products is similar to that of native dengue E. In addition, this blot demonstrates that under reducing conditions, 80%E ZipperII runs as a monomer similar in size to 80%E ZipperI and 80%E Bundle. This is again consistent with formation of a disulfide bond between the cysteines located near the carboxy-terminal end of the leucine zipper helices.

Example 7

Recombinant Dimeric 80%E Products are Recognized by Conformationally-Sensitive Monoclonal Antibodies

The reactivity of the recombinant dimeric 80%E products with conformationally-sensitive monoclonal antibodies (MAbs) was assessed using indirect immunofluorescence assays (IFA). Transfected S2 cells were plated onto slides and fixed with ice-cold acetone. The cells were then treated with various polyclonal and

monoclonal antibodies diluted in PBS containing 20% FBS. After washing away unbound antibody, bound antibody was detected by reacting the cells with fluorescein isothiocyante-labeled goat anti-mouse immunoglobulin and observing on a fluorescent microscope after excitation at 470 nm. Cells transfected with the Linked 80%E Dimer, 80%E Bundle, 80%E ZipperI, and 80%E ZipperII were efficiently recognized by the conformationally sensitive MAbs 9D12 and 4G2 (Henchal et al., 1992; Mason et al., 1989). In addition all transfectants were recognized by MAb 5A2 which binds to a linear epitope located in the domain B region of E (Megret et al., 1992). These data suggest that these recombinant, dimeric products are antigenically similar to native viral E and therefore may serve as a useful vaccine immunogen.

Example 8

Induction of dengue virus neutralizing antibodies upon immunization of mice with secreted dimeric 80%E produced by transfected S2 cells

S2 cells expressing Linked 80%E Dimer, 80%E Bundle, 80%E ZipperI, and 80%E ZipperII were cultured in serum-free medium (supplemented IPL-41; Gibco BRL) and induced by addition of $CuSO_4$ to a final concentration of 0.2 mM in the culture medium (see example 5 for more detail on culture conditions). The cells were maintained in inducing medium for seven days prior to harvesting. The cells were removed by centrifugation at 1000 X G in a Beckman TJ-6 refrigerated centrifuge and the media were filtered through a 0.2 μm cellulose acetate filter (Nalgene). The media containing the recombinant dimeric 80%E products were concentrated approximately ten fold and buffer-exchanged with PBS. The total protein concentration of the medium was determined using a dye binding assay (Biorad). Balb/c mice (Jackson Laboratories) were immunized intraperitoneally with 100 μg total protein of each concentrated medium (of which only ~ 5-10% was the dengue protein)in Freund's complete adjuvant. The mice were boosted twice, at two week intervals, with 50 μg of each medium in Freund's incomplete adjuvant. Ten days following the last boost the animals were sacrificed and their blood obtained for testing.

The sera from the immunized mice were tested for the presence of antibodies which bind to recombinant DEN-2 80%E using an indirect ELISA assay. Briefly,

plates were coated with purified, recombinant DEN-2 80%E, blocked with bovine serum albumin (BSA), and serial dilutions of the mouse sera were then incubated with the coating antigen. Alkaline phosphatase-labeled goat anti-mouse IgG was used as the secondary detecting antibody, and the color development upon addition of an alkaline phosphatase chromogenic substrate was monitored. The ELISA titer is the reciprocal of the highest dilution of serum which resulted in an optical density two-fold above background (reactivity of the serum against BSA only).

The sera were also tested for virus neutralizing antibodies using a plaque reduction neutralization test (PRNT). In the PRNT assay, the mouse sera were serially diluted in Eagles minimal essential medium (EMEM; BioWhittaker) supplemented with 10% FBS (Hyclone) and mixed with 100 plaque forming units of Vero-adapted DEN-2 virus (from Robert Putnak, WRAIR). After allowing one hour for neutralization of the virus, the mixtures were plated onto susceptible monkey kidney monolayers (Vero cells, from Robert Putnak, WRAIR) plated in EMEM containing 10% FBS in 6 well tissue culture dishes (Costar). After allowing two hours for the virus to bind, the cells were overlaid with 0.9% agarose (Fisher) in EMEM supplemented with 5% FBS. Viral cytopathic effect was allowed to develop for 6-7 days and the viral plaques were stained with 0.012% neutral red (Sigma) in 1% agarose. The number of plaques in each cluster were counted and compared to a no-serum viral control. The $PRNT_{80}$ titer was the reciprocal of the highest dilution of serum which resulted in at least 80% reduction in the number of plaques compared to the no-serum viral control. Results from the ELISA and PRNT assays are summarized in Table 1. All of the media induced a virus-binding and neutralizing response in the mice demonstrating that all of the dimeric 80%E immunogens are capable of functioning as efficient immunogens.

Table 1
Induction of Anti-DEN-2 Immune Response in Mice Immunized with Crude Media Containing Dimeric 80%E Products

Mouse Number	Immunogen	ELISA Titer	$PRNT_{80}$ Titer
179-1	Linked 80%E Dimer crude medium 100 µg Freund's adjuvant	25,600	800
179-2		1600	10
179-3		6400	1000
179-4		6400	400
179-5		25,600	4000
180-1	80%E Bundle crude medium 100 µg Freund's adjuvant	1600	1000
180-2		6400	400
180-3		6400	400
180-4		1600	200
180-5		6400	4000
181-1	80%E ZipperI crude medium 100 µg Freund's adjuvant	25,600	8000
181-2		6400	200
181-3		6400	2000
181-4		6400	2000
181-5		1600	200
182-1	80%E ZipperII crude medium 100 µg Freund's adjuvant	25,600	800
182-2		1600	100
182-3		400	100
182-4		1600	200
182-5		6400	1000
177-1	PBS Iscomatrix Adjuvant	<100	<10
177-2		<100	<10
177-3		<100	<10
177-4		<100	<10
177-5		<100	<10

Example 9

The Secreted, Recombinant Dimeric 80%E Products can be Efficiently Purified Using Immunoaffinity Chromatography

The conformationally sensitive MAb 9D12 has been previously used in our laboratory to efficiently purify monomeric DEN-2 80%E. This MAb binds to a conformational epitope in the domain B region (amino acids 296-395) of DEN-2 E. MAb 9D12 was covalently coupled to a HiTrap column (Pharmacia) and used to immunoaffinity-purify each of the recombinant dimeric 80%E molecules, Linked 80%E Dimer, 80%E ZipperI, 80%E ZipperII, and 80%E Bundle. Crude media containing the products was applied to the column and unbound material removed by

extensive washing with phosphate-buffered saline (PBS). Bound material was eluted with 0.1 M Glycine HCl pH 2.5 and immediately neutralized with 1.0 M Phosphate pH 7.4. The products were concentrated and buffer exchanged into PBS prior to analysis on SDS-PAGE gels. Each of the products was efficiently purified using this column (Figure 11). In all cases the vast majority of the dimeric 80%E bound to the column and was efficiently eluted in a relatively small volume. Thus, this method offers an efficient means of generating purified dimeric 80%E products for animal testing.

Example 10

Induction of high titer dengue virus-neutralizing antibodies upon immunization of mice with purified, secreted dimeric 80%E

Culture media from S2 cells expressing Linked 80%E Dimer, 80%E Bundle, 80%E ZipperI, and 80%E ZipperII, prepared as described in Example 8, were used as a source of antigen for additional mouse immunization studies. Each of the products was purified using immunoaffinity chromatography (IAC) as described in Example 9.

Purified Linked 80%E Dimer, 80%E ZipperI, 80%E ZipperII, and 80%E Bundle products were assayed using a quantitative Sandwich ELISA assay, SDS-PAGE analysis, and Western blotting. In the Sandwich ELISA assay MAb 9D12 was coated onto the plates, which were then blocked with BSA. Serial dilutions of a quantitated DEN-2 domain B standard or the products to be assayed were applied in triplicate to each well. Bound antigen was detected using a polyclonal rabbit anti-DEN-2 domain B antibody and horseradish peroxidase-conjugated anti-rabbit immunoglobulin. Chromogenic substrate for the horseradish peroxidase was added and the color development monitored. The absorbance generated by the test antigen was compared to the standard curve and the amount of antigen present in domain B equivalents is determined. To convert from domain B equivalents to dimeric 80%E, the weight ratio (~ 4.5 for most of the products), determined by comparing the relative molecular weight of the dimeric 80%E to domain B and dividing by the number of

domain B regions present in the dimeric 80%E product, was used. Each purified dimeric product was quantitated using this assay for mouse immunizations.

Balb/c mice (Jackson Laboratories) were immunized with 1 μg of each purified, secreted dimeric 80%E product. The immunizations were given subcutaneously using Iscomatrix (Iscotech) adjuvant. Two immunizations were given at 4 week intervals. Ten days following the final immunization the mice were sacrificed and their sera tested for virus binding and neutralizing antibodies by ELISA and PRNT as described in example 8. The results are summarized in Table 2. As is clearly evident, all of the dimeric 80%E products induced a high-titer virus neutralizing response. These titers are higher than any titers previously reported in the literature and suggest that these dimeric 80%E products are exceptionally effective vaccine candidates.

Table 2
Induction of Anti-DEN-2 Immune Response in Mice Immunized with Purified Recombinant Dimeric 80%E Products

Mouse Number	Immunogen	ELISA Titer	$PRNT_{80}$ Titer
173-1	IAC-pure	102,400	4000
173-2	Linked 80%E Dimer	102,400	8000
173-3	1 µg	102,400	8000
173-4	Iscomatrix	102,400	4000
173-5	Adjuvant	102,400	4000
185-1	IAC-pure	102,400	32,000
185-2	80%E Bundle	25,600	4000
185-3	1 µg	25,600	4000
185-4	Iscomatrix	25,600	16,000
185-5	Adjuvant	102,400	2000
174-1	IAC-pure	6400	200
174-2	80%E ZipperI	409,600	4000
174-3	1 µg	102,400	8000
174-4	Iscomatrix	102,400	16,000
174-5	Adjuvant	102,400	8000
175-1	IAC-pure	102,400	8000
175-2	80%E ZipperII	25,600	2000
175-3	1 µg	102,400	16,000
175-4	Iscomatrix	102,400	8000
175-5	Adjuvant	102,400	4000
176-1	IAC-pure	102,400	4000
176-2	80%E	102,400	16,000
176-3	1 µg	25,600	8000
176-4	Iscomatrix	25,600	4000
176-5	Adjuvant	102,400	4000
177-1		<100	<10
177-2	PBS	<100	<10
177-3	Iscomatrix	<100	<10
177-4	Adjuvant	<100	<10
177-5		<100	<10

Claims

1. A vaccine for the protection of a subject against infection by a *Flavivirus*, wherein said vaccine comprises a therapeutically effective amount of a dimeric 80%E, said dimeric 80%E having been secreted as a recombinantly produced protein from eucaryotic cells.

2. The vaccine of claim 1 wherein said dimeric 80%E is selected from the group consisting of: linked 80%E dimer; 80%E ZipperI; 80%E ZipperII; and 80%E Bundle.

3. The vaccine of claim 2 wherein the linked 80%E dimer is a truncated envelope protein of serotype DEN-1.

4. The vaccine of claim 2 wherein the linked 80%E dimer is a truncated envelope protein of serotype DEN-2.

5. The vaccine of claim 1 wherein the linked 80%E dimer is a truncated envelope protein of serotype DEN-3.

6. The vaccine of claim 1 wherein the linked 80%E dimer is a truncated envelope protein of serotype DEN-4.

7. A multivalent vaccine for the protection of a subject against infection by a *Flavivirus*, wherein said vaccine comprises a therapeutically effective amount of a first dimeric 80%E product of one flaviviral serotype; a second dimeric 80%E product of a second flaviviral serotype; a third dimeric 80%E product of a third flaviviral serotype; and a fourth dimeric 80%E product of a fourth flaviviral serotype; wherein all dimeric 80%E products have been secreted as recombinantly produced protein from a eucaryotic cell.

8. A vaccine of claim 7 wherein said dimeric 80%E products are envelope proteins of serotypes selected from the group consisting of: DEN-1; DEN-2; DEN-3; and DEN-4.

9. A composition of antibodies consisting essentially of antibodies generated in a mammalian subject administered an immunogenic amount of the vaccine of claim 1.

10. A composition of antibodies consisting essentially of antibodies generated in a mammalian subject administered an immunogenic amount of the vaccine of claim 2.

11. A composition of antibodies consisting essentially of antibodies generated in a mammalian subject administered an immunogenic amount of the vaccine of claim 7.

12. A composition of antibodies consisting essentially of antibodies generated in a mammalian subject administered an immunogenic amount of the vaccine of claim 8.

13. An immortalized B cell line, wherein said B cells have been generated in response to the administration to a mammalian subject of an immunogenic amount of the vaccine of claim 1.

14. An immortalized B cell line, wherein said B cells have been generated in response to the administration to a mammalian subject of an immunogenic amount of the vaccine of claim 2.

15. An immortalized B cell line, wherein said B cells have been generated in response to the administration to a mammalian subject of an immunogenic amount of the vaccine of claim 7.

16. An immortalized B cell line, wherein said B cells have been generated in response to the administration to a mammalian subject of an immunogenic amount of the vaccine of claim 8.

17. Monoclonal antibodies secreted by the immortalized B cell line of claim 13.

18. Monoclonal antibodies secreted by the immortalized B cell line of claim 14.

19. Monoclonal antibodies secreted by the immortalized B cell line of claim 15.

20. Monoclonal antibodies secreted by the immortalized B cell line of claim 16.

21. The vaccine of claim 1 wherein said *Flavivirus* is a dengue virus.

22. The vaccine of claim 2 wherein said *Flavivirus* is a dengue virus.

23. The vaccine of claim 7 wherein said *Flavivirus* is a dengue virus.

24. The vaccine of claim 8 wherein said *Flavivirus* is a dengue virus.

25. A method to protect a subject against a *Flavivirus*, which method comprises administering to a subject in need of such protection an effective amount of the vaccine of any one of claims 1-8.

26. An immunodiagnostic for the detection of a *Flavivirus*, wherein said immunodiagnostic comprises, a dimeric 80%E having been secreted as a recombinantly produced protein from *Drosophila* cells.

27. The immunodiagnostic of claim 26, wherein said *Flavivirus* is a dengue virus.

28. A multivalent immunodiagnostic for the detection of a *Flavivirus*, wherein said immunodiagnostic comprises, a first dimeric 80%E product of one flaviviral serotype; a second dimeric 80%E product of a second flaviviral serotype; a third dimeric 80%E product of a third flaviviral serotype; and a fourth dimeric 80%E product of a fourth flaviviral serotype; wherein all dimeric 80%E products have been secreted as recombinantly produced protein from a eucaryotic cell.

29. The immunodiagnostic of claim 28, wherein said *Flavivirus* is a dengue virus.

30. An immunodiagnostic kit for the detection of a *Flavivirus*, in a test subject wherein said immunodiagnostic kit comprises:

a) dimeric 80%E wherein, said dimeric 80%E has been secreted as recombinantly produced protein from *Drosophila* cells;

b) a suitable solid support phase coated with said dimeric 80%E; and

c) labeled antibodies immunoreactive to antibodies from said test subject.

31. An immunodiagnostic kit for the detection of a *Flavivirus*, in a test subject wherein said immunodiagnostic kit comprises:

a) a first dimeric 80%E product of one flaviviral serotype; a second dimeric 80%E product of a second flaviviral serotype; a third dimeric 80%E product of a third flaviviral serotype; and a fourth dimeric 80%E product of a fourth flaviviral

serotype; wherein all dimeric 80%E products have been secreted as recombinantly produced protein from a eucaryotic cell;

b) a suitable solid support phase coated with said dimeric 80%E products; and

c) labeled antibodies immunoreactive to antibodies from said test subject.

32. The immunodiagnostic kit of claim 30, wherein said *Flavivirus* is a dengue virus.

33. The immunodiagnostic kit of claim 31, wherein said *Flavivirus* is a dengue virus.

34. A vector host recombinant DNA expression system, which comprises:

a) a suitable eucaryotic host cell;

b) a suitable recombinant DNA expression vector;

c) DNA encoding dimeric 80%E, operably linked and under the control of a suitable promoter; and

d) said DNA encoding dimeric 80%E operably linked to a secretory signal leader sequence.

35. The vector host recombinant DNA system of claim 34, wherein said dimeric 80%E is selected from the group consisting of: linked 80%E dimer; 80%E ZipperI; 80%E ZipperII; and 80%E Bundle.

36. The vector host recombinant DNA system of claim 35, wherein said eucaryotic host cell is a *Drosophila* cell.

37. A DNA sequence encoding dimeric 80%E.

38. The DNA sequence of claim 37, wherein the dimeric 80%E is selected from the group consisting of: linked 80%E dimer; 80%E ZipperI; 80%E ZipperII; and 80%E Bundle.

5

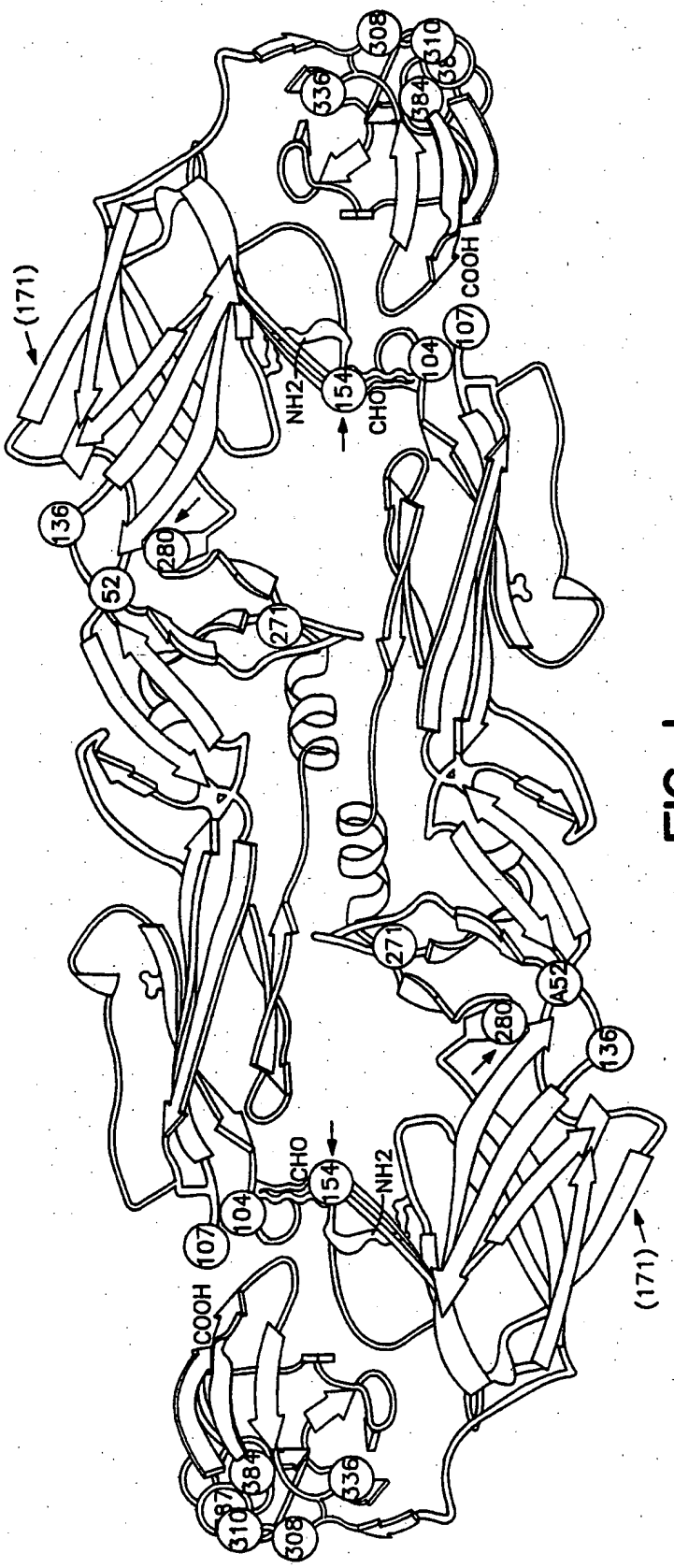
508
310
384
336
(171)
COOH
107
104
NH2
154
CHO
136
280
52
27
271
A52
280
136
CHO
154
NH2
104
107
COOH
(171)
384
336
310
508

FIG. 1

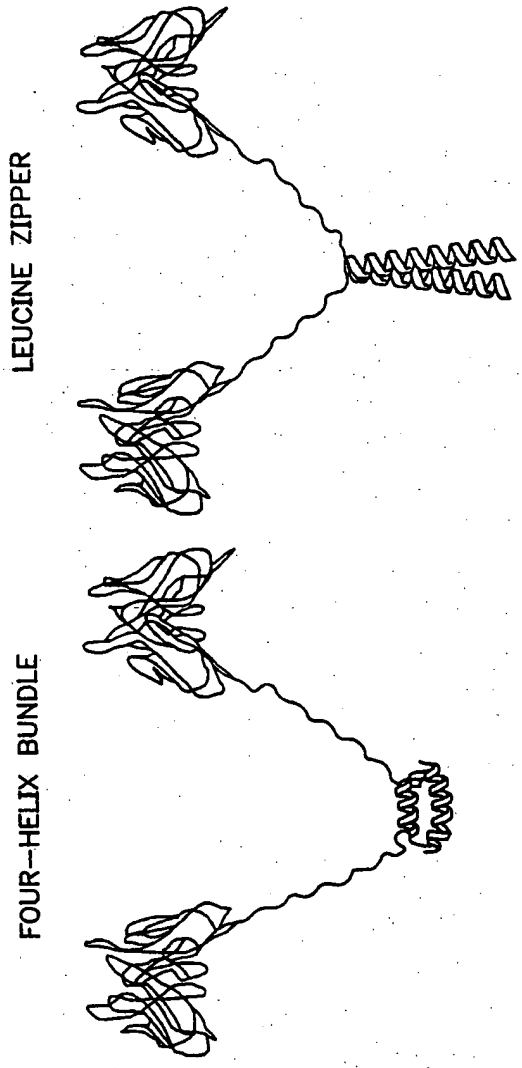

FIG. 2B

FIG. 2A

```
                                                *
97                                  ATG AATAACCAACG GAAAAAGGCG AGAAACACGC
                                    Met AsnAsnGlnArg LysLysAla ArgAsnThr>
                                    ◆ Capsid

131 CTTTCAATAT GCTGAAACGC GAGAGAAACC GCGTGTCAAC TGTACAACAG TTGACAAAGA
   ProPheAsnMet LeuLysArg GluArgAsn ArgValSerThr ValGlnGln LeuThrLys>

191 GATTCTCACT TGGAATGCTG CAGGGACGAG GACCACTAAA ATTGTTCATG GCCCTGGTGG
   ArgPheSerLeu GlyMetLeu GlnGlyArg GlyProLeuLys LeuPheMet AlaLeuVal>

251 CATTCCTTCG TTTCCTAACA ATCCCACCAA CAGCAGGGAT ATTAAAAAGA TGGGGAACAA
   AlaPheLeuArg PheLeuThr IleProPro ThrAlaGlyIle LeuLysArg TrpGlyThr>

311 TTAAAAAATC AAAGGCTATT AATGTTCTGA GAGGCTTCAG GAAAGAGATT GGAAGGATGC
   IleLysLysSer LysAlaIle AsnValLeu ArgGlyPheArg LysGluIle GlyArgMet>

371 TGAATATCTT AAACAGGAGA CGTAGAACTG CAGGCATGAT CATCATGCTG ATTCCAACAG
   LeuAsnIleLeu AsnArgArg ArgArgThr AlaGlyMetIle IleMetLeu IleProThr>

431 TGATGGCGTT TCATCTGACC ACACGCAACG GAGAACCACA CATGATCGTC AGTAGACAAG
   ValMetAlaPhe HisLeuThr ThrArgAsn GlyGluProHis MetIleVal SerArgGln>
            ◆ PreMembrane

491 AAAAAGGGAA AAGCCTTCTG TTTAAGACAA AGGACGGCAC GAACATGTGT ACCCTCATGG
   GluLysGlyLys SerLeuLeu PheLysThr LysAspGlyThr AsnMetCys ThrLeuMet>

551 CCATGGACCT TGGTGAGTTG TGTGAAGACA CAATCACGTA TAAATGTCCC TTTCTCAAGC
   AlaMetAspLeu GlyGluLeu CysGluAsp ThrIleThrTyr LysCysPro PheLeuLys>

611 AGAACGAACC AGAAGACATA GATTGTTGGT GCAACTCCAC GTCCACATGG GTAACTTATG
   GlnAsnGluPro GluAspIle AspCysTrp CysAsnSerThr SerThrTrp ValThrTyr>

671 GGACATGTAC CACCACAGGA GAGCACAGAA GAGAAAAAAG ATCAGTGGCG CTTGTTCCAC
   GlyThrCysThr ThrThrGly GluHisArg ArgGluLysArg SerValAla LeuValPro>
                                                ◆ Membrane

731 ACGTGGGAAT GGGATTGGAG ACACGAACTG AAACATGGAT GTCATCAGAA GGGGCCTGGA
   HisValGlyMet GlyLeuGlu ThrArgThr GluThrTrpMet SerSerGlu GlyAlaTrp>

791 AACATGCCCA GAGAATTGAA ACTTGGATTC TGAGACATCC AGGCTTTACC ATAATGGCCG
   LysHisAlaGln ArgIleGlu ThrTrpIle LeuArgHisPro GlyPheThr IleMetAla>

851 CAATCCTGGC ATACACCATA GGAACGACGC ATTTCCAAAG AGTCCTGATA TTCATCCTAC
   AlaIleLeuAla TyrThrIle GlyThrThr HisPheGlnArg ValLeuIle PheIleLeu>

911 TGACAGCCAT CGCTCCTTCA ATGACAATGC GCTGCATAGG AATATCAAAT AGGGACTTTG
   LeuThrAlaIle AlaProSer MetThrMet ArgCysIleGly IleSerAsn ArgAspPhe>
                                    ◆ Envelope

971 TGGAAGGAGT GTCAGGAGGG AGTTGGGTTG ACATAGTTTT AGAACATGGA AGTTGTGTGA
   ValGluGlyVal SerGlyGly SerTrpVal AspIleValLeu GluHisGly SerCysVal>
```

FIG. 3A

```
1031 CGACGATGGC AAAAAATAAA CCAACACTGG ACTTTGAACT GATAAAAACA GAAGCCAAAC
    ThrThrMetAla LysAsnLys ProThrLeu AspPheGluLeu IleLysThr GluAlaLys>

1091 AACCCGCCAC CTTAAGGAAG TACTGTATAG AGGCTAAACT GACCAACACG ACAACAGACT
    GlnProAlaThr LeuArgLys TyrCysIle GluAlaLysLeu ThrAsnThr ThrThrAsp>

1151 CGCGCTGCCC AACACAAGGG GAACCCACCC TGAATGAAGA GCAGGACAAA AGGTTTGTCT
    SerArgCysPro ThrGlnGly GluProThr LeuAsnGluGlu GlnAspLys ArgPheVal>

1211 GCAAACATTC CATGGTAGAC AGAGGATGGG GAAATGGATG TGGATTATTT GGAAAAGGAG
    CysLysHisSer MetValAsp ArgGlyTrp GlyAsnGlyCys GlyLeuPhe GlyLysGly>

1271 GCATCGTGAC CTGTGCCATG TTCACATGCA AAAAGAACAT GGAGGGAAAA ATTGTGCAGC
    GlyIleValThr CysAlaMet PheThrCys LysLysAsnMet GluGlyLys IleValGln>

1331 CAGAAAACCT GGAATACACT GTCGTTATAA CACCTCATTC AGGGGAAGAA CATGCAGTCG
    ProGluAsnLeu GluTyrThr ValValIle ThrProHisSer GlyGluGlu HisAlaVal>

1391 GAAATGACAC AGGAAAACAT GGTAAAGAAG TCAAGATAAC ACCACAGAGC TCCATCACAG
    GlyAsnAspThr GlyLysHis GlyLysGlu ValLysIleThr ProGlnSer SerIleThr>

1451 AGGCGGAACT GACAGGCTAT GGCACTGTTA CGATGGAGTG CTCTCCAAGA ACGGGCCTCG
    GluAlaGluLeu ThrGlyTyr GlyThrVal ThrMetGluCys SerProArg ThrGlyLeu>

1511 ACTTCAATGA GATGGTGTTG CTGCAAATGA AAGACAAAGC TTGGCTGGTG CACAGACAAT
    AspPheAsnGlu MetValLeu LeuGlnMet LysAspLysAla TrpLeuVal HisArgGln>

1571 GGTTCCTAGA CCTACCGTTG CCATGGCTGC CCGGAGCAGA CACACAAGGA TCAAATTGGA
    TrpPheLeuAsp LeuProLeu ProTrpLeu ProGlyAlaAsp ThrGlnGly SerAsnTrp>

1631 TACAGAAAGA GACACTGGTC ACCTTCAAAA ATCCCCATGC GAAAAAACAG GATGTTGTTG
    IleGlnLysGlu ThrLeuVal ThrPheLys AsnProHisAla LysLysGln AspValVal>

1691 TCTTAGGATC CCAAGAGGGG GCCATGCATA CAGCACTCAC AGGGGCTACG GAAATCCAGA
    ValLeuGlySer GlnGluGly AlaMetHis ThrAlaLeuThr GlyAlaThr GluIleGln>

1751 TGTCATCAGG AAACCTGCTG TTCACAGGAC ATCTTAAGTG CAGGCTGAGA ATGGACAAAT
    MetSerSerGly AsnLeuLeu PheThrGly HisLeuLysCys ArgLeuArg MetAspLys>

1811 TACAACTTAA AGGGATGTCA TACTCCATGT GCACAGGAAA GTTTAAAGTT GTGAAGGAAA
    LeuGlnLeuLys GlyMetSer TyrSerMet CysThrGlyLys PheLysVal ValLysGlu>

1871 TAGCAGAAAC ACAACATGGA ACAATAGTCA TTAGAGTACA ATATGAAGGA GACGGCTCTC
    IleAlaGluThr GlnHisGly ThrIleVal IleArgValGln TyrGluGly AspGlySer>

                *
1931 CATGCAAGAT CCCTTTTGAG ATAATGGATC TGGAAAAAAG ACATGTTTTG GGCCGCCTGA
    ProCysLysIle ProPheGlu IleMetAsp LeuGluLysArg HisValLeu GlyArgLeu>

  *                                   *
1991 TCACAGTCAA CCCAATTGTA ACAGAAAAGG ACAGCCCAGT CAACATAGAA GCAGAACCTC
    IleThrValAsn ProIleVal ThrGluLys AspSerProVal AsnIleGlu AlaGluPro>

2051 CATTCGGAGA CAGCTACATC ATCATAGGAG TGGAACCAGG ACAATTGAAG CTGGACTGGT
    ProPheGlyAsp SerTyrIle IleIleGly ValGluProGly GlnLeuLys LeuAspTrp>
```

FIG. 3B

2111 TCAAGAAAGG AAGTTCCATC GGCCAAATGT TTGAGACAAC AATGAGGGGA GCGAAAAGAA
PheLysLysGly SerSerIle GlyGlnMet PheGluThrThr MetArgGly AlaLysArg>

2171 TGGCCATTTT GGGCGACACA GCCTGGGATT TTGGATCTCT GGGAGGAGTG TTCACATCAA
MetAlaIleLeu GlyAspThr AlaTrpAsp PheGlySerLeu GlyGlyVal PheThrSer>

2231 TAGGAAAGGC TCTCCACCAG GTTTTTGGAG CAATCTACGG GGCTGCTTTC AGTGGGGTCT
IleGlyLysAla LeuHisGln ValPheGly AlaIleTyrGly AlaAlaPhe SerGlyVal>

2291 CATGGACTAT GAAGATCCTC ATAGGAGTTA TCATCACATG GATAGGAATG AACTCACGTA
SerTrpThrMet LysIleLeu IleGlyVal IleIleThrTrp IleGlyMet AsnSerArg>

2351 GCACATCACT GTCTGTGTCA CTGGTATTAG TGGGAATCGT GACACTGTAC TTGGGAGTTA
SerThrSerLeu SerValSer LeuValLeu ValGlyIleVal ThrLeuTyr LeuGlyVal>

2411 TGGTGCAGGC CGATAGTGGT TGCGTTGTGA GCTGGAAGAA CAAAGAACTA AAATGTGGCA
MetValGlnAla AspSerGly CysValVal SerTrpLysAsn LysGluLeu LysCysGly>
◆ NS1

2471 GTGGAATATT CGTCACAGAT AACGTGCATA CATGGACAGA ACAATACAAG TTCCAACCAG
SerGlyIlePhe ValThrAsp AsnValHis ThrTrpThrGlu GlnTyrLys PheGlnPro>

2531 AATCCCCTTC AAAACTGGCT TCAGCCATCC AGAAAGCTCA TGAAGAGGGC ATCTGTGGAA
GluSerProSer LysLeuAla SerAlaIle GlnLysAlaHis GluGluGly IleCysGly>

2591 TCCGCTCAGT AACAAGACTG GAAAATCTTA TGTGGAAACA AATAACATCA GAATTGAATC
IleArgSerVal ThrArgLeu GluAsnLeu MetTrpLysGln IleThrSer GluLeuAsn>

2651 ATATTCTATC AGAAAATGAA GTGAAACTGA CCATCATGAC AGGAGACATC AAAGGAATCA
HisIleLeuSer GluAsnGlu ValLysLeu ThrIleMetThr GlyAspIle LysGlyIle>

2711 TGCAGGTAGG AAAACGATCT CTGCGGCCTC AACCCACTGA GTTGAGGTAT TCATGGAAAA
MetGlnValGly LysArgSer LeuArgPro GlnProThrGlu LeuArgTyr SerTrpLys>

2771 CATGGGGTAA AGCGAAAATG CTCTCCACAG AACTCCATAA TCAGACCTTC CTCATTGATG
ThrTrpGlyLys AlaLysMet LeuSerThr GluLeuHisAsn GlnThrPhe LeuIleAsp>

2831 GTCCCGAAAC AGCAGAATGC CCCAACACAA ACAGAGCTTG GAATTCACTA GAAGTTGAGG
GlyProGluThr AlaGluCys ProAsnThr AsnArgAlaTrp AsnSerLeu GluValGlu>

2891 ACTACGGCTT TGGAGTATTC ACTACCAATA TATGGCTAAG ATTGAGAGAA AAGCAGGATG
AspTyrGlyPhe GlyValPhe ThrThrAsn IleTrpLeuArg LeuArgGlu LysGlnAsp>

2951 CATTTTGTGA CTCAAAACTC ATGTCAGCGG CCATAAAGGA CAACAGAGCC GTCCATGCTG
AlaPheCysAsp SerLysLeu MetSerAla AlaIleLysAsp AsnArgAla ValHisAla>

3011 ATATGGGTTA TTGGATAGAA AGCGCACTCA ATGATACATG GAAGATAGAG AAAGCTTCTT
AspMetGlyTyr TrpIleGlu SerAlaLeu AsnAspThrTrp LysIleGlu LysAlaSer>

3071 TCATTGAAGT CAAAAGTTGC CACTGGCCAA AGTCACACAC TCTATGGAGT AATGGAGTGC
PheIleGluVal LysSerCys HisTrpPro LysSerHisThr LeuTrpSer AsnGlyVal>

3131 TAGAAAGCGA GATGGTAATT CCAAAGAATT TCGCTGGACC AGTGTCACAA CATAATAACA
LeuGluSerGlu MetValIle ProLysAsn PheAlaGlyPro ValSerGln HisAsnAsn>

FIG. 3C

```
3191 GACCAGGCTA TCACACACAA ACAGCAGGAC CTTGGCATCT AGGCAAGCTT GAGATGGACT
    ArgProGlyTyr HisThrGln ThrAlaGly ProTrpHisLeu GlyLysLeu GluMetAsp>

3251 TTGATTTCTG CGAAGGGACT ACAGTGGTGG TAACCGAGGA CTGTGGAAAC AGAGGGCCCT
    PheAspPheCys GluGlyThr ThrValVal ValThrGluAsp CysGlyAsn ArgGlyPro>

3311 CTTTAAGAAC AACCACTGCC TCAGGAAAAC TCATAACGGA ATGGTGTTGT CGATCTTGCA
    SerLeuArgThr ThrThrAla SerGlyLys LeuIleThrGlu TrpCysCys ArgSerCys>

3371 CACTACCACC ACTAAGATAC AGAGGTGAGG ATGGATGCTG GTACGGGATG GAAATCAGAC
    ThrLeuProPro LeuArgTyr ArgGlyGlu AspGlyCysTrp TyrGlyMet GluIleArg>

3431 CATTGAAAGA GAAAGAAGAA AATCTGGTCA GTTCTCTGGT CACAGCC
    ProLeuLysGlu LysGluGlu AsnLeuVal SerSerLeuVal ThrAla
```

FIG. 3D

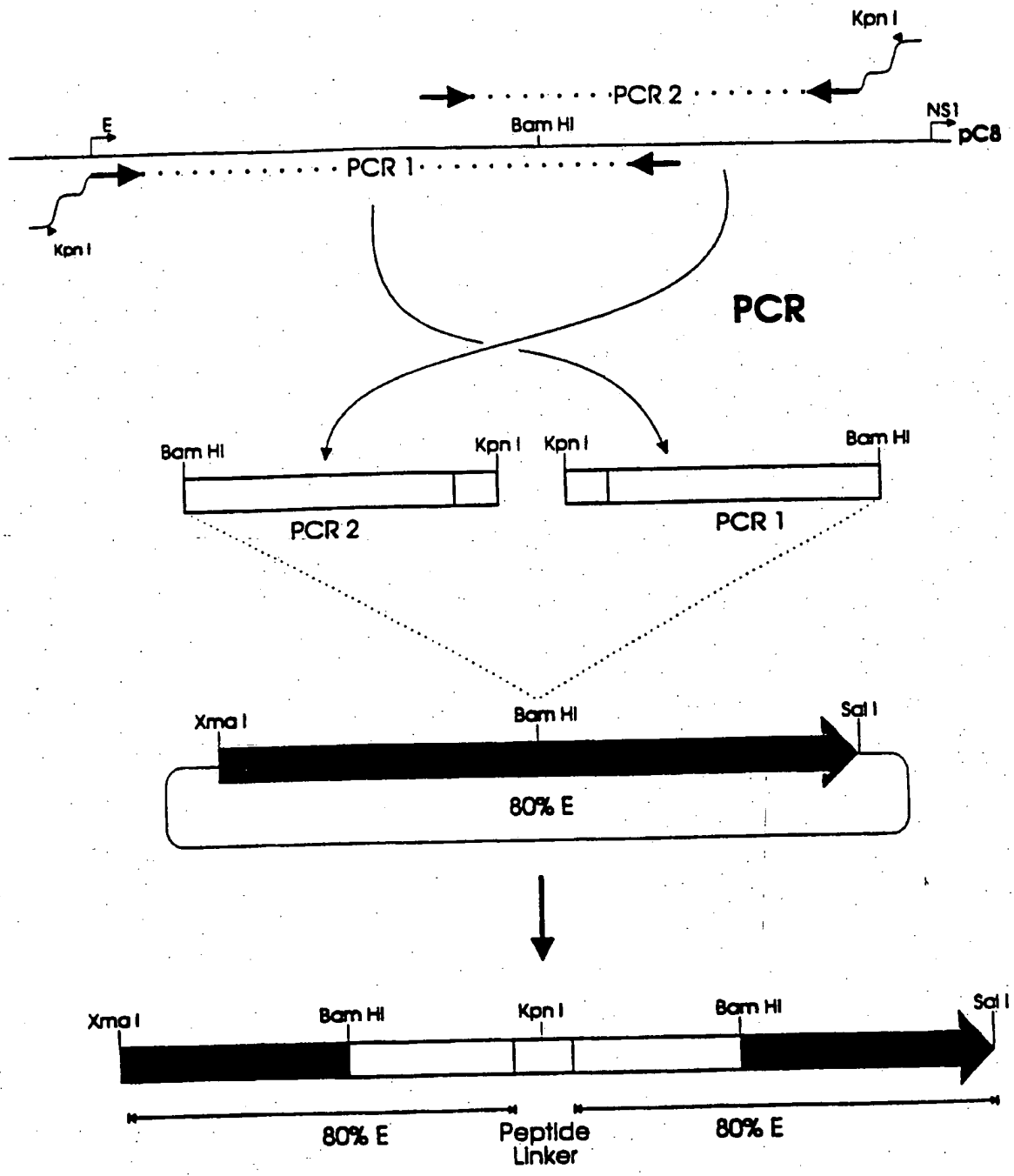
Kpn I
PCR 2
NS1
pC8
E
Bam HI
PCR 1
Kpn I
PCR
Bam HI
Kpn I
Kpn I
Bam HI
PCR 2
PCR 1
Xma I
Bam HI
Sal I
80% E
Xma I
Bam HI
Kpn I
Bam HI
Sal I
80% E
Peptide Linker
80% E

FIG. 4

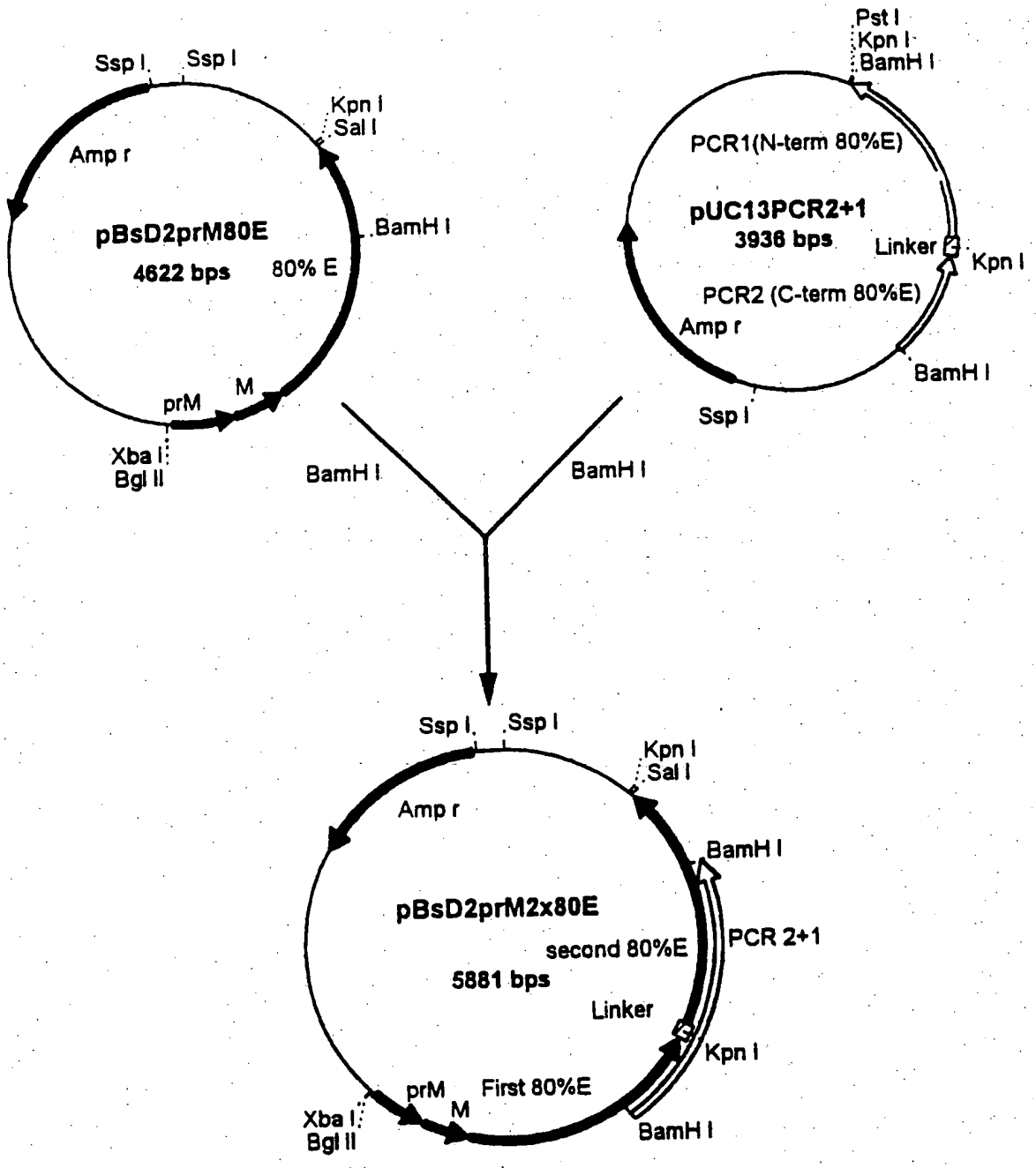
Ssp I
Ssp I
Kpn I
Sal I
Amp r
pBsD2prM80E
4622 bps
80% E
BamH I
prM
M
Xba I
Bgl II
Pst I
Kpn I
BamH I
PCR1(N-term 80%E)
pUC13PCR2+1
3936 bps
Linker
Kpn I
PCR2 (C-term 80%E)
Amp r
BamH I
Ssp I
BamH I
BamH I
Ssp I
Ssp I
Kpn I
Sal I
Amp r
BamH I
pBsD2prM2x80E
second 80%E
PCR 2+1
5881 bps
Linker
Kpn I
prM
First 80%E
M
Xba I
Bgl II
BamH I

FIG. 5

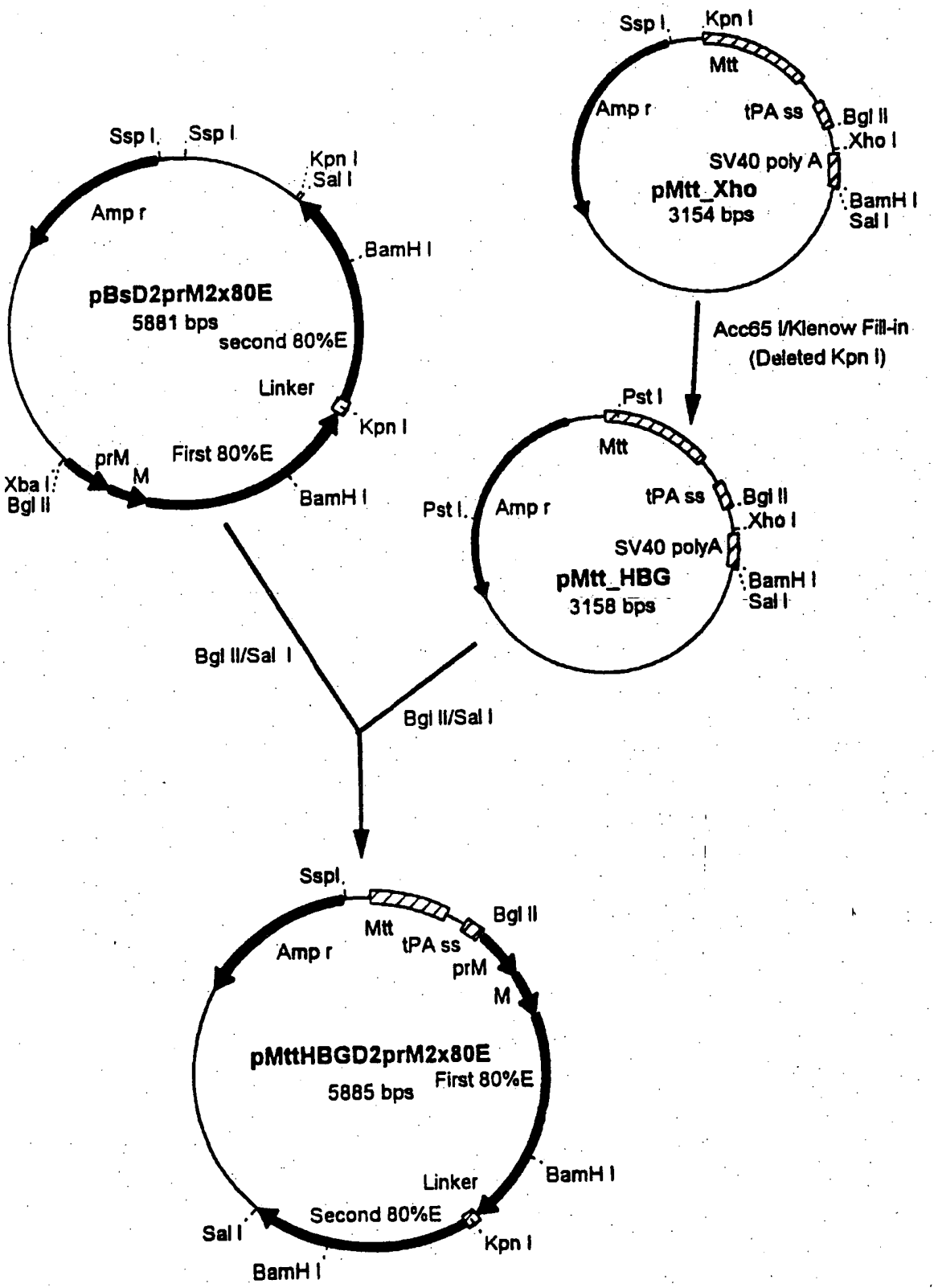
Ssp I
Kpn I
Mtt
Amp r
tPA ss
Bgl II
Xho I
SV40 poly A
pMtt_Xho
3154 bps
BamH I
Sal I
Acc65 I/Klenow Fill-in
(Deleted Kpn I)
Ssp I
Ssp I
Kpn I
Sal I
Amp r
BamH I
pBsD2prM2x80E
5881 bps
second 80%E
Linker
Kpn I
prM
M
First 80%E
Xba I
Bgl II
BamH I
Pst I
Mtt
Pst I
Amp r
tPA ss
Bgl II
Xho I
SV40 polyA
pMtt_HBG
3158 bps
BamH I
Sal I
Bgl II/Sal I
Bgl II/Sal I
SspI
Mtt
Bgl II
Amp r
tPA ss
prM
M
pMttHBGD2prM2x80E
First 80%E
5885 bps
Linker
BamH I
Second 80%E
Sal I
Kpn I
BamH I

FIG. 6

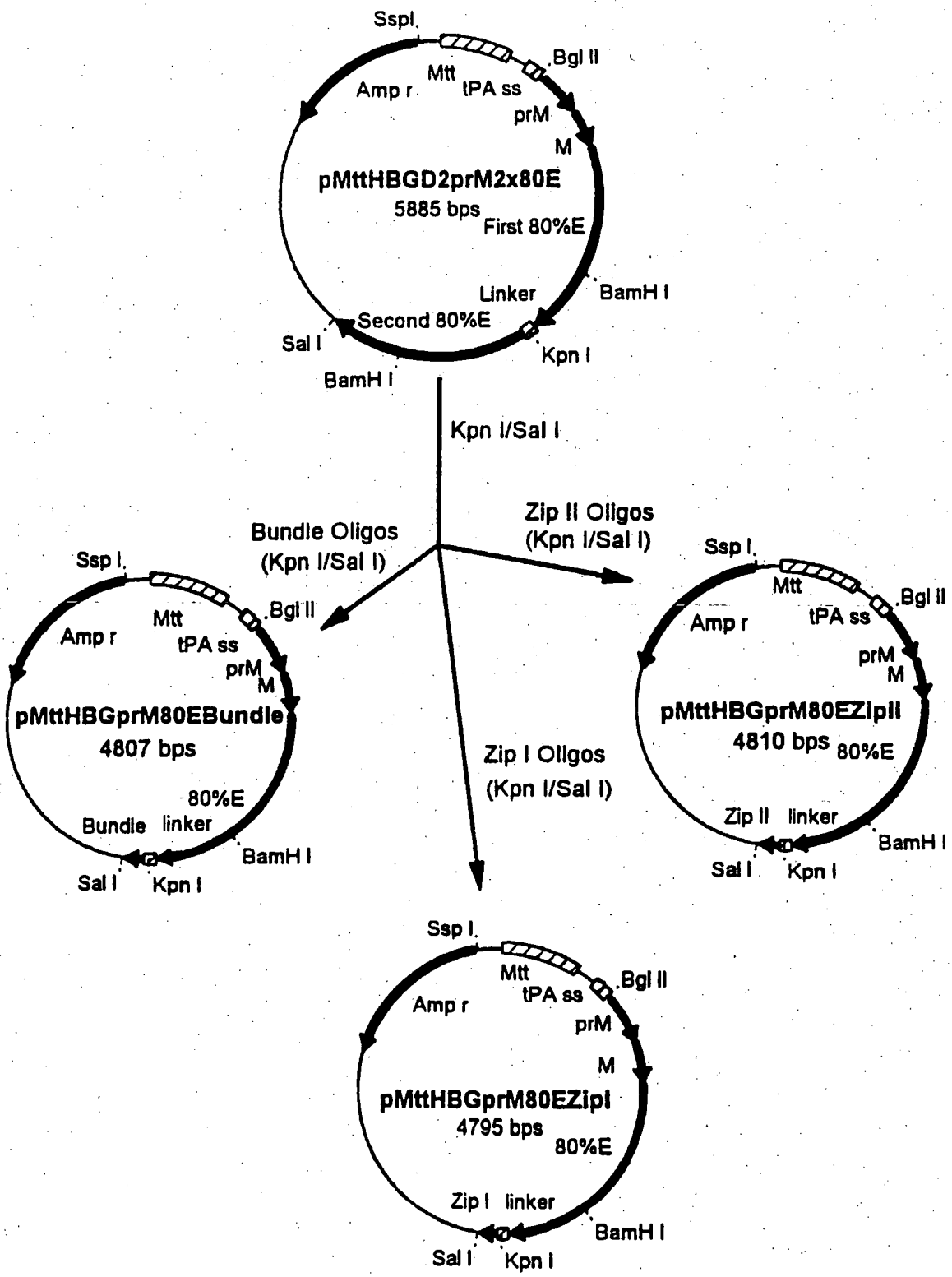
SspI
Bgl II
Mtt
Amp r
tPA ss
prM
M
pMttHBGD2prM2x80E
5885 bps
First 80%E
Linker
BamH I
Second 80%E
Sal I
Kpn I
BamH I
Kpn I/Sal I
Bundle Oligos
(Kpn I/Sal I)
Zip II Oligos
(Kpn I/Sal I)
Zip I Oligos
(Kpn I/Sal I)
Ssp I
Mtt
Bgl II
Amp r
tPA ss
prM
M
pMttHBGprM80EBundle
4807 bps
80%E
Bundle linker
BamH I
Sal I
Kpn I
Ssp I
Mtt
Bgl II
Amp r
tPA ss
prM
M
pMttHBGprM80EZipII
4810 bps 80%E
Zip II linker
BamH I
Sal I
Kpn I
Ssp I
Mtt
Bgl II
Amp r
tPA ss
prM
M
pMttHBGprM80EZipI
4795 bps
80%E
Zip I linker
BamH I
Sal I
Kpn I

FIG. 7

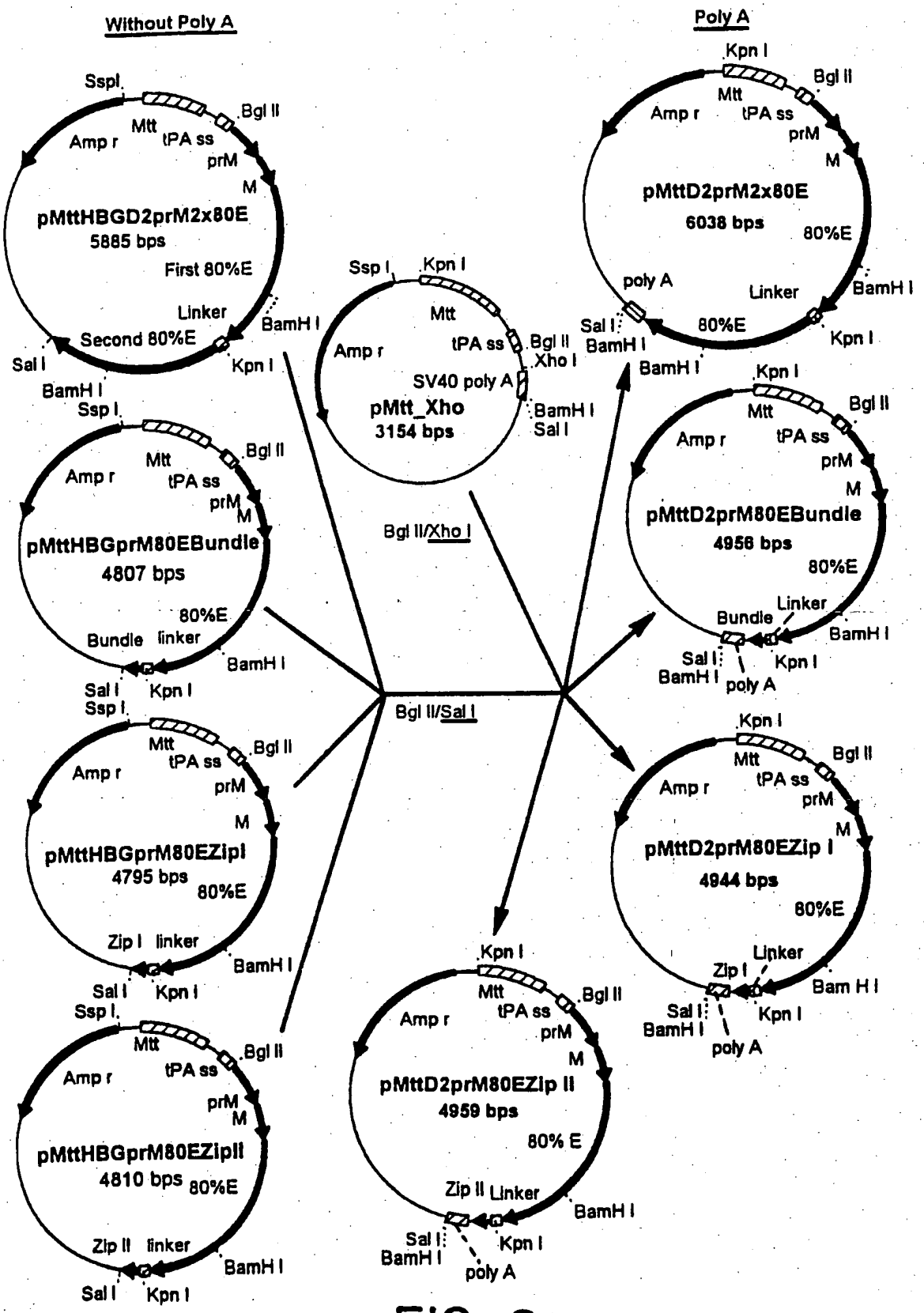
Without Poly A
Poly A
pMttHBGD2prM2x80E
5885 bps
pMttHBGprM80EBundle
4807 bps
pMttHBGprM80EZipI
4795 bps
pMttHBGprM80EZipII
4810 bps
pMtt_Xho
3154 bps
pMttD2prM2x80E
6038 bps
pMttD2prM80EBundle
4956 bps
pMttD2prM80EZip I
4944 bps
pMttD2prM80EZip II
4959 bps
Bgl II/Xho I
Bgl II/Sal I
Sspl
Ssp I
Kpn I
Bgl II
Mtt
tPA ss
prM
M
Amp r
First 80%E
Second 80%E
Linker
BamH I
Sal I
Xho I
SV40 poly A
poly A
80%E
Bundle
linker
Zip I
Zip II
Sal I
Kpn I

FIG. 8

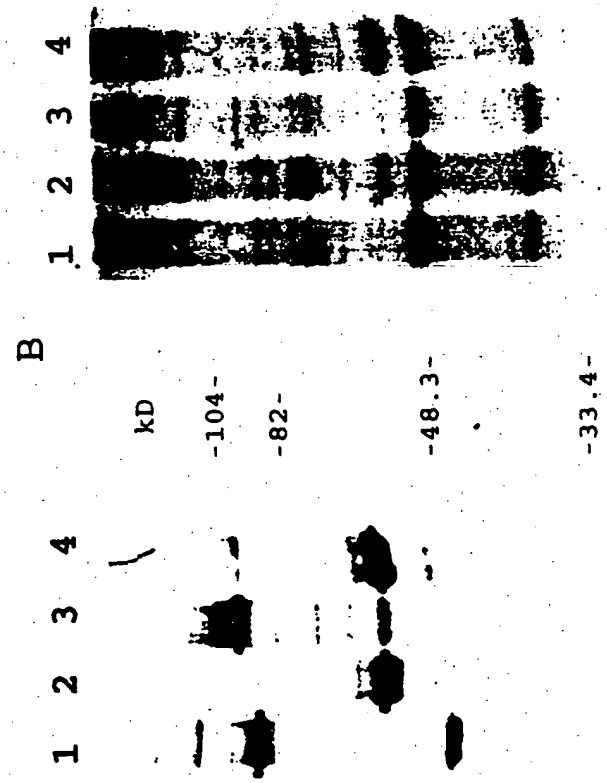

Figure 9. SDS-PAGE Analysis of Secreted Dimeric 80%E Products. Crude media from cells transformed with the dimeric 80%E constructs were separated on non-reducing 10% SDS-PAGE gels and detected by immunoprobing(Panel A) or Coomassie blue staining (Panel B). Lanes: 1) Linked 80%E Dimer media; 2) 80%E ZipperI media; 3) 80%E ZipperII media; 4) 80%E Bundle media. The locations and sizes of molecular weight markers in kilodaltons are indicated. The location of products on the Coomassie blue stained gel is indicated by *.

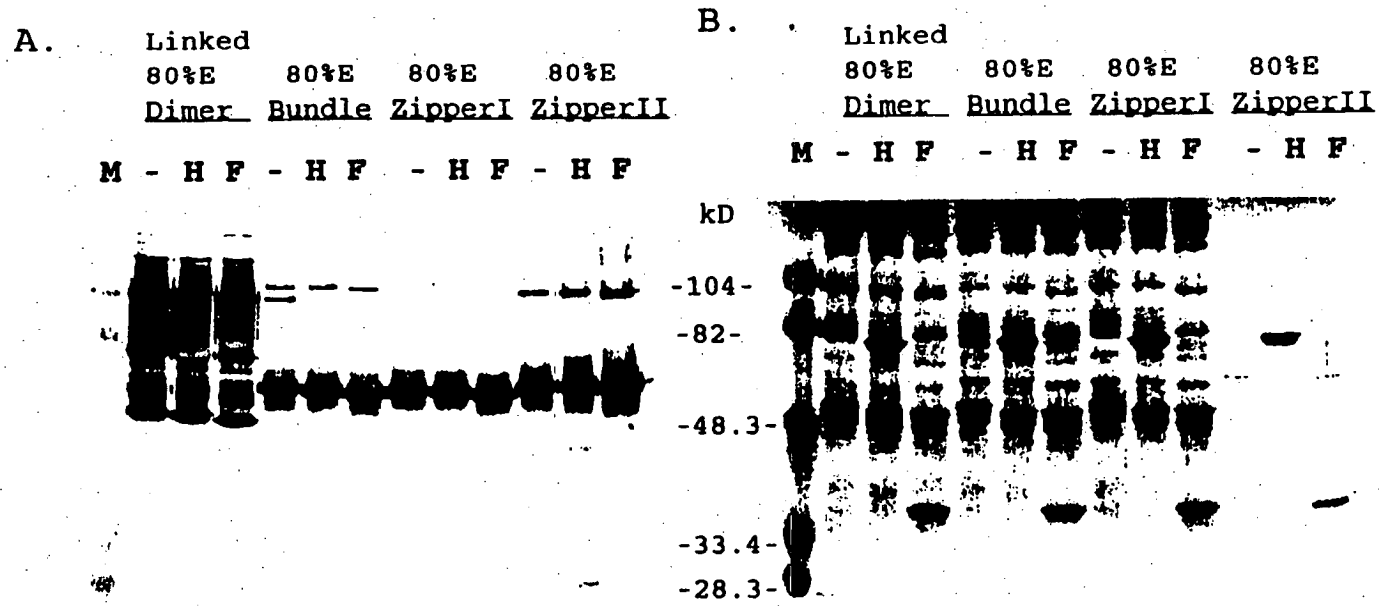

Figure 10. Endoglycosidase Digestion and SDS-PAGE Analysis of Secreted Dimeric 80%E Products. The secreted dimeric 80%E products were denatured and reduced and treated with no enzyme (-), Endoglycosidase H (H), or PNGase F (F), prior to separation on reducing SDS-PAGE gels. The products were detected by immunoprobing (Panel A) or Coomassie blue staining (Panel B). The locations and sizes of molecular weight markers in kilodaltons are indicated.

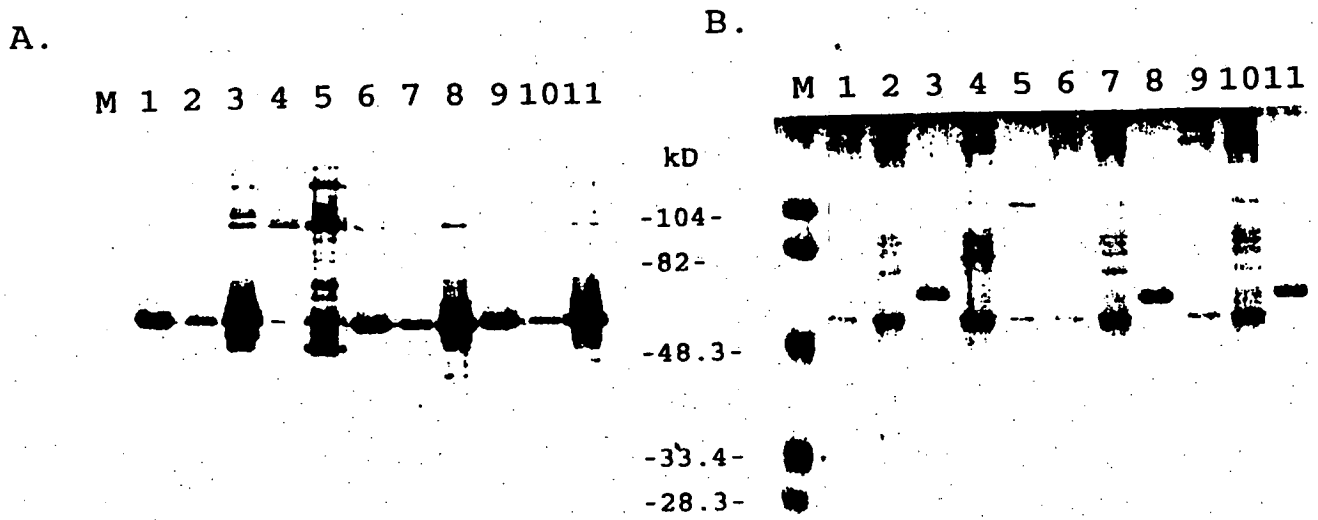

Figure 11. Immunoaffinity Purification of Secreted Dimeric 80%E Products. A conformationally sensitive monoclonal antibody was used to prepare an immunoaffinity column for purification of the secreted dimeric 80%E products. Crude media containing the products, flow through concentrated 10-fold, and the purified products were separated on reducing SDS-PAGE gels and detected by immunoprobing (Panel A) or Coomassie blue staining (Panel B). Lanes: 1) 80%E Bundle medium; 2) 80%E Bundle flow through; 3) purified 80%E Bundle; 4) Linked 80%E Dimer flow through; 5) purified Linked 80%E Dimer; 6) 80%E ZipperI medium; 7) 80%E ZipperI flow through; 8) purified 80%E ZipperI; 9) 80%E ZipperII medium; 10) 80%E ZipperII flow through; 11) purified 80%E ZipperII. The locations and sizes of molecular weight markers in kilodaltons are indicated.